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PRINCIPAL INVESTIGATOR: Seung-Uon Shin, Ph.D.

CONTRACTING ORGANIZATION: University of Miami
Miller School of Medicine
Miami, FL 33136

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Seung-Uon Shin, Ph.D.

Email: mvalero@med.miami.edu

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14. ABSTRACT
The anti-angiogenic protein endostatin demonstrated considerable anti-tumor activity in animal models. However, limited anti-tumor activity has been observed in human Phase I/II trials. Trastuzumab has activity in HER2+ breast cancer used alone or in combination with chemotherapy. Prior studies using an anti-HER2 antibodymurine endostatin fusion demonstrated enhanced anti-tumor activity compared to anti-HER2 antibody or endostatin given alone or in combination. We generated two anti-HER2 human endostatin fusion proteins by fusing human wild type or a mutant form of human endostatin (huEndo-P125A) to the 3' end of a humanized anti-HER2 IgG3 antibody. HuEndo-P125A antibody fusion protein (αHER2-huEndo-P125A) inhibited VEGF and bFGF induced endothelial cell proliferation, and capillary formation in vitro, to a greater degree than wild type endostatin fusion protein (αHER2-huEndo), endostatin alone, or anti-HER2 antibody (αHER2 IgG3). Treatment of SKBR-3 breast cancer xenografts with anti-HER2 IgG3-huEndo-P125A fusion resulted in complete regression, and improved survival, compared to either αHER2 IgG3, human endostatin, or anti-HER2 IgG3-huEndo treated mice. αHER2-huEndo fusion proteins specifically targeted tumors expressing HER2 in mice simultaneously implanted with murine mammary tumor cell line EMT6 and EMT6 engineered to express HER2 antigen (EMT6-HER2). αHER2 huEndo-P125A fusion antibody showed enhanced anti-angiogenic and anti-tumor activity and inhibited EMT6-HER2 growth more effectively than huEndo ($p = 0.003$), or αHER2-huEndo ($p = 0.004$). Targeting anti-angiogenic proteins using antibody fusion proteins could improve clinical activity of anti-HER2 antibody and endostatin alike, and provides a versatile approach that could be applied to other tumor targets with alternative antibody specificities or using other antiangiogenic domains.

15. SUBJECT TERMS
Immunotherapy, Angiogenesis, Chemotherapy, Antibody, Endostatin, Breast Cancer, HER2/neu

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BREAST CANCER THERAPY USING ANTIBODY-ENDOSTATIN FUSION PROTEINS

INTRODUCTION

Angiogenesis is critical for growth of primary tumor and for the development of metastasis. Antiangiogenic therapy with agents such as endostatin is under active investigation. Early human trials showed endostatin to be safe, but minimal activity has been observed.[1-3] Dosage and schedules may have been suboptimal, and/or late stage disease may not be responsive to recombinant human endostatin. HER2 is overexpressed in 30% of breast cancer and phase II trials of Herceptin demonstrated an 11% response rate in HER2+ patients with metastatic breast disease.[4-6] Combining Herceptin with chemotherapy enhances anti-tumor activity resulting in an objective response rate of 60 % or greater in several phase III trials.[5,7,8] To produce a more effective form of Herceptin and improve the efficacy of endostatin, we have constructed an anti-HER2 IgG3-C_H3-endostatin fusion protein by joining murine endostatin to the 3' end of humanized anti-HER2 IgG3.[9] Preliminary data using an antibody-murine endostatin fusion protein suggests enhanced effectiveness of anti-HER2 IgG3-endostatin may be due to longer endostatin half-life and the selective targeting of endostatin to tumor by anti-HER2 antibody due to the presence of a fused antiangiogenic factor.[9]

The objective of the proposal is to develop and test novel antibody-fusion proteins with specific ability to deliver antiangiogenic factors to tumors by linking an antiangiogenic factor, human endostatin, with the targeting specificity of an antibody directed against HER2 in order to direct localization of endostatin to the tumor site. Application of the strategy in humans will require careful evaluation of antibody fusion protein antigenicity and might benefit from use of a human endostatin fusion domain. If the antibody-endostatin fusion protein is specifically targeted to the surface of tumor cells, it will be more effective because of retained antibody effector functions, effects on HER2 signaling, and improved ability to inhibit neovascularization in a tumor specific fashion.

To achieve our goals, we set up three specific aims. I. Design and synthesize two variant antibody-human endostatin (huEndo) fusion proteins (anti-HER2 IgG3-Hinge-huEndo and anti-HER2 IgG3-C_H3-huEndo) directed against HER2, which differ in the Fc region and its ability to mediate antibody effector functions (Fig. 1). II. Test the antiangiogenic activity of anti-HER2 antibody-human endostatin fusion protein(s) *in vitro* and *in vivo*. III. Study the antibody-endostatin fusion proteins *in vivo* for effects on tumor growth in animal tumor and/or human xenograft models.

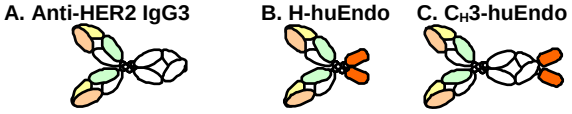


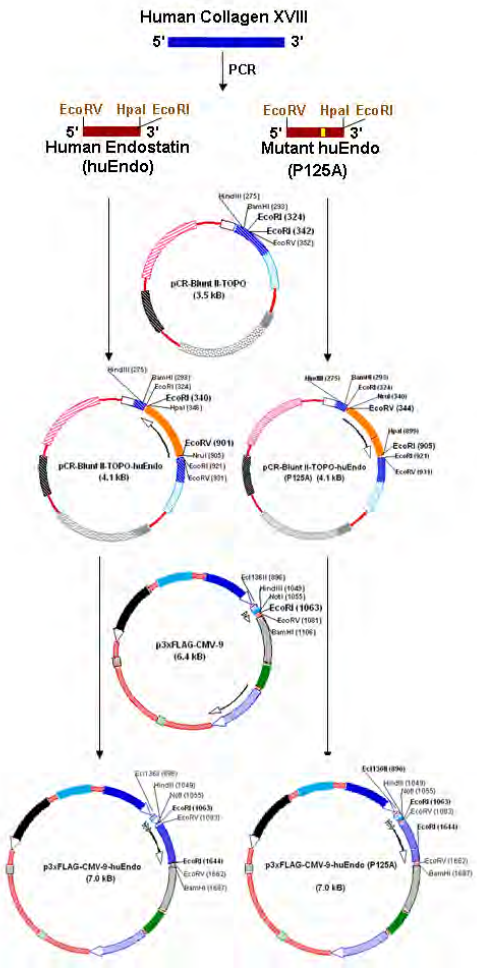
Fig. 1. Schematic diagram of anti-HER2 IgG3-human endostatin fusion proteins. Endostatin domain in orange.

BODY

Specific Aim I. Design and synthesize two variant antibody-human endostatin (huEndo) fusion proteins (anti-HER2 IgG3-Hinge-huEndo (αHER2-H-Endo) and anti-HER2 IgG3-C_H3-huEndo (αHER2-C_H3-Endo)) directed against HER2, which differ in the Fc region and its ability to mediate antibody effector functions.

Task 1. Construction and expression of anti-HER2 IgG3-H-huEndo and anti-HER2 IgG3-C_H3-huEndo fusion proteins (Months 1-6).

Construction and expression of human endostatin and a mutant



Human endostatin (huEndo) gene originated from the human collagen, type XVIII, alpha 1 gene by PCR using primers 5'-CCCCTCGCGATATCACAGCCACCGCGACTTCCAGCCG and 5'-CCCCGAATTCGTT AACCCTTGGAGGCAGTCATGAAGC. The PCR products were cloned into pCR-Blunt II-TOPO vector and sequenced. After sequencing, clones containing wild-type human endostatin and single-point mutant clones at a position 125 (An alanine residue was substituted for proline at position 125 by site-directed mutagenesis using primer 5'-GGCTCGGACGCCAACGGGCGC; P125A.) were identified. A point mutation in human endostatin at position 125 (proline to alanine) has improved endothelial cell binding and antiangiogenic activity.[10-12] The wild-type (huEndo) and the mutant human endostatin (huEndo-P125A) genes were subsequently cloned into p3xFLAG-CMV-9 vector under the control of the human cytomegalovirus promoter, in which preprotrypsin leader sequence precedes the FLAG sequence (Fig. 2).[13-15] The FLAG-tagged human endostatin will be distinguishable from the endogenous endostatin *in vivo*. p3xFLAG-CMV-9-huEndo and p3xFLAG-CMV-9-huEndo-P125A vectors were stably transfected into COS cells and their expression of endostatin is being investigated now.

Fig. 2. Cloning of human endostatin and a mutant endostatin (P125A). PCR cloned human endostatin genes were subcloned into pBlunt II-TOPO vector, and were subsequently cloned into 3xFLAG-CMV-9 vector to express secreted human endostatin tagged with FLAG.

Construction of anti-HER2 IgG3-human endostatin fusion proteins

The subcloned huEndo and huEndo-P125A genes were ligated in frame to the carboxyl end of the heavy chain constant domain (Hinge or C_H3) of human IgG3 in the vector pAT135 as described previously (Fig. 3)[16] and the endostatin heavy chain constant region was then joined to an anti-HER2 variable region of a recombinant humanized monoclonal antibody 4D5-8 (HER2, trastuzumab; Genentech) in the expression vector (pSV2-his) containing HisD gene for eukaryotic selection (Fig. 4).[17,18]

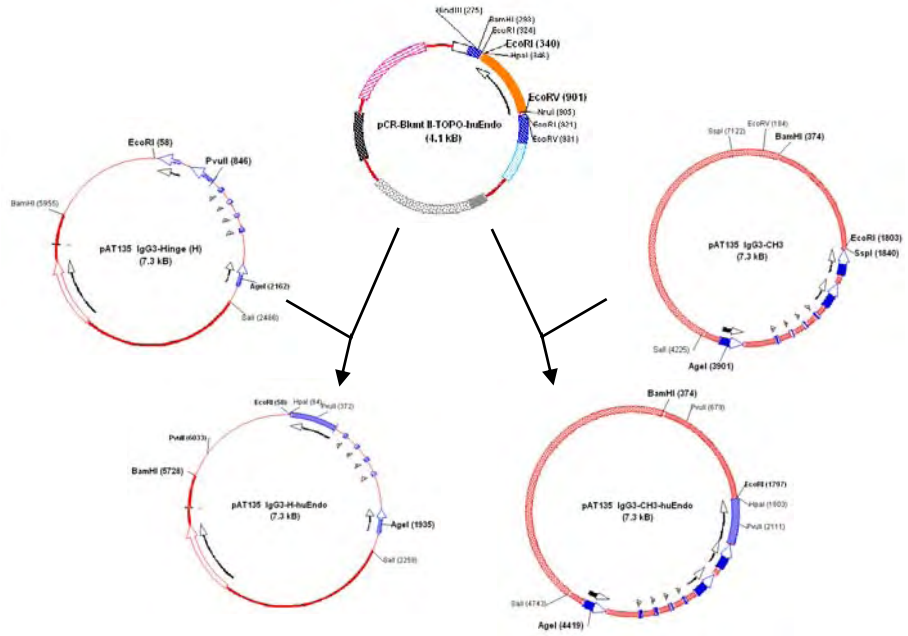


Fig. 3. Schematic diagram of cloning steps of human endostatin into human IgG3 in pAT135 vector.

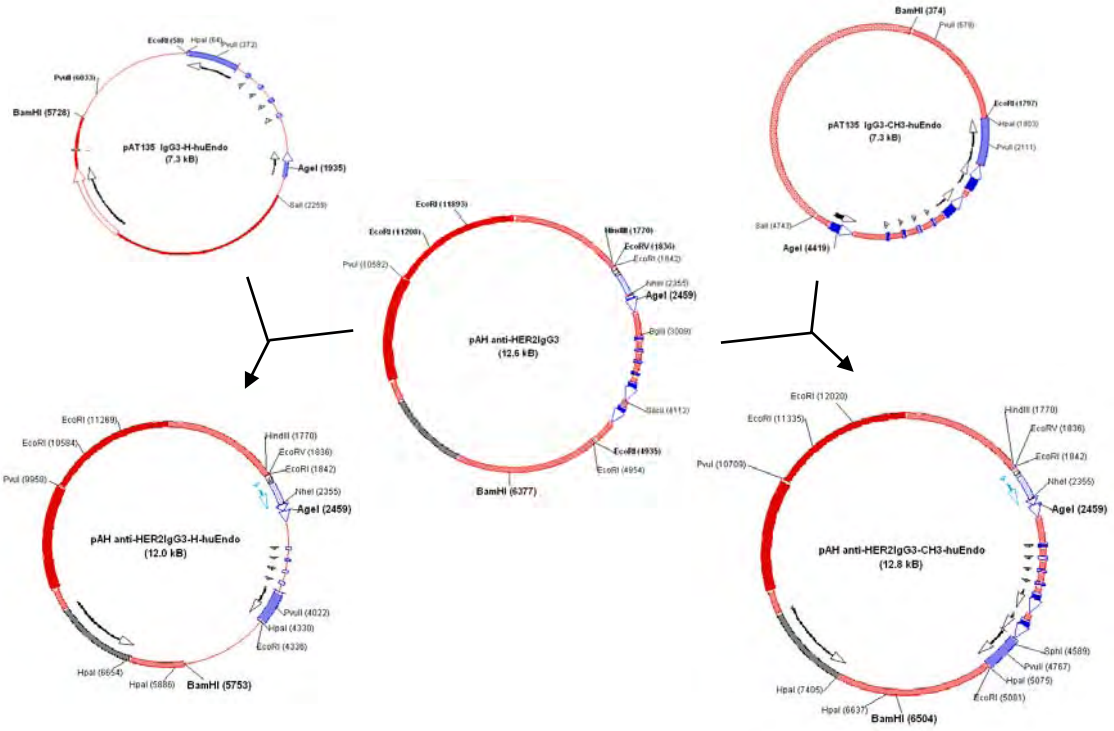


Fig. 4. Schematic diagram of cloning steps of IgG3-huEndo into the expression vector.

Expression of anti-HER2 IgG3-human endostatin fusion proteins

The anti-HER2 IgG3-huEndo fusion protein constructs were stably transfected into SP2/0 or P3X63Ag8.653 myeloma cells stably expressing the anti-HER2 kappa light chain to assemble the entire anti-HER2 IgG3-huEndo fusion proteins as described previously (Fig. 5).[19]

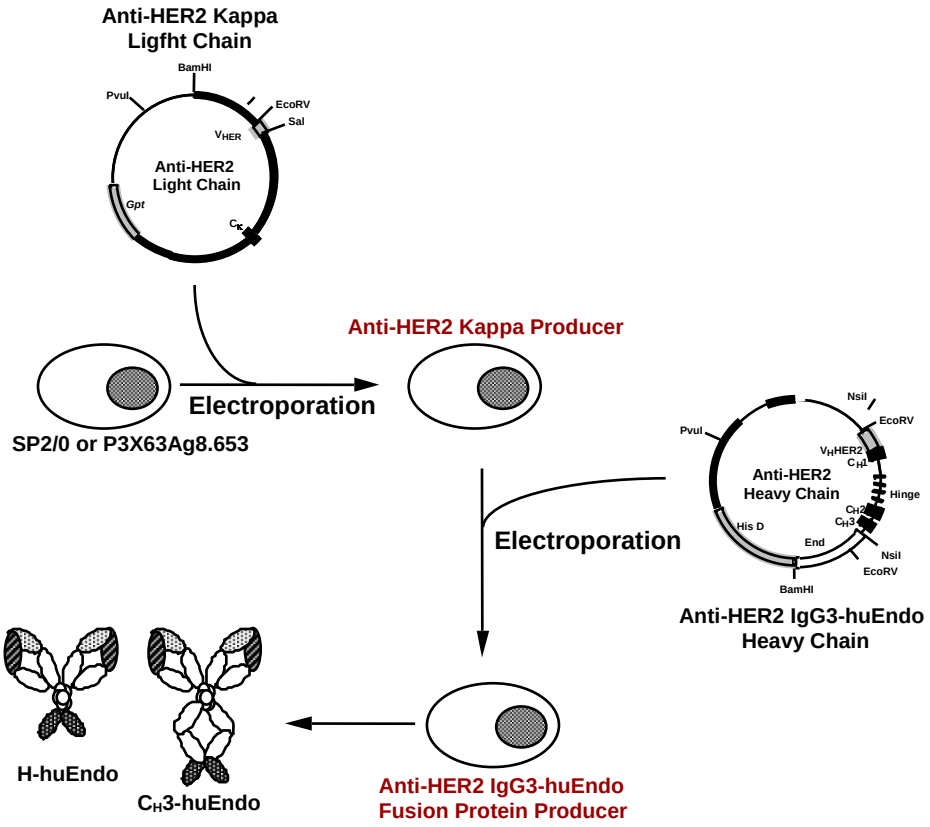


Fig.5. Producing transfectants expressing anti-HER2 IgG3-huEndo fusion proteins. Anti-HER2 kappa light chain producer was generated by electroporation, and subsequently the light chain producer was transfected with anti-HER2 IgG3-huEndo fusion heavy chain gene. The huEndo fusion protein producers were identified through ELISA.

Task 2. Produce anti-HER2 H-huEndo and anti-HER2 IgG3-C_H3-huEndo fusion proteins and endostatin (Months 1-24).

Production of endostatin fusion proteins and endostatin

We generated the αHER2-H-Endo and αHER2-C_H3-Endo fusion proteins. We obtained high quantity and good quality of the C_H3-Endo fusion proteins (Fig.6, both the native and the mutant type). However, we experienced difficulties with purifying the αHER2-H-Endo using Protein L columns that strongly bind the immunoglobulin light chain, because most of the purified proteins were light chain dimers (50 kDa) instead of the αHER2-H-Endo (170 kDa) fusion protein. We tried to then purify αHER2-H-Endo using either Protein G or Protein A affinity column, but we obtained very low concentrations of H-Endo and the αHER2-H-Endo were co-purified with bovine IgG from culture supernatant with 1% fetal clone sera (serum with very low concentration of bovine IgG). We stopped production of αHER2-H-Endo.

Human native endostatin (Endo) and a mutant endostatin (A point mutation at position 125 (proline to alanine) has improved endothelial cell binding and antiangiogenic activity.[10-12]) were secreted from CHO cells Since human endostatin was tagged with FLAG, we detected secreted human endostatin molecules using anti-flag antibody by Western blotting (Fig. 7). Size of human endostatin and human mutant endostatin are bigger than murine endostatin, because human endostatin was tagged with 3x FLAG and murine endostatin tagged with 1x FLAG. We tried to purify endostatin with either anti-FLAG antibody column or heparin sulfate column, but we recovered very little quantities of both human endostatin for our studies. Thus, we decided to do our experiment with commercially available human endostatin, while we continued on solving the problems with purification.

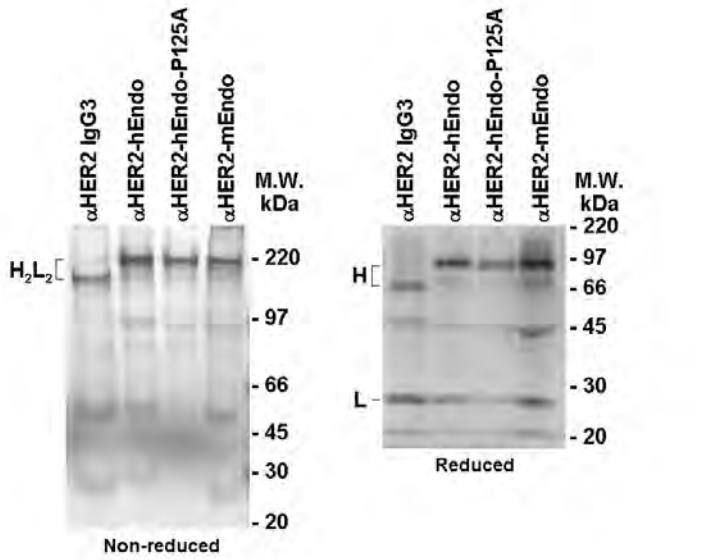


Fig. 6. Expression of anti-HER2 IgG3-C_H3-endostatin fusion proteins (the wild type and the mutant type P125A). Secreted human endostatin fusion proteins were labeled with [^{35}S]methionine and immunoprecipitated with Protein A and analyzed under non-reducing and reducing conditions. Control anti-HER2 IgG3-C_H3-murine endostatin fusion was used as control.

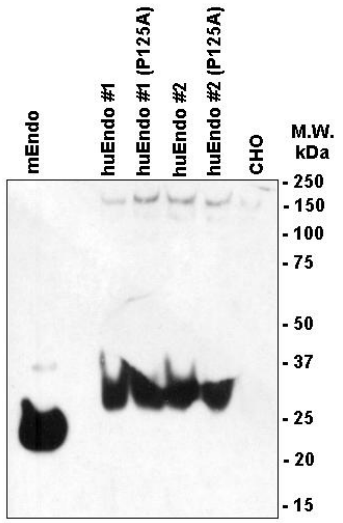


Fig. 7. Expression of human endostatin and a mutant endostatin (P125A). Secreted human endostatin tagged with FLAG was identified with anti-FLAG antibody from the CHO culture supernatant. Murine endostatin tagged with FLAG was used as conrol.

Binding ability to HER2 and antibody-dependent cell-mediated cytotoxicity

To investigate whether the endostatin fusion proteins bind to the HER2 antigen on tumors, frozen section of CT26 and CT26-HER2 tumors were incubated with endostatin fusion proteins and stained with secondary anti-human IgG antibody-HRP, which was used against DAB for visualization (Fig. 8).[9] The wild type and the mutant type endostatin fusion proteins specifically recognized the HER2

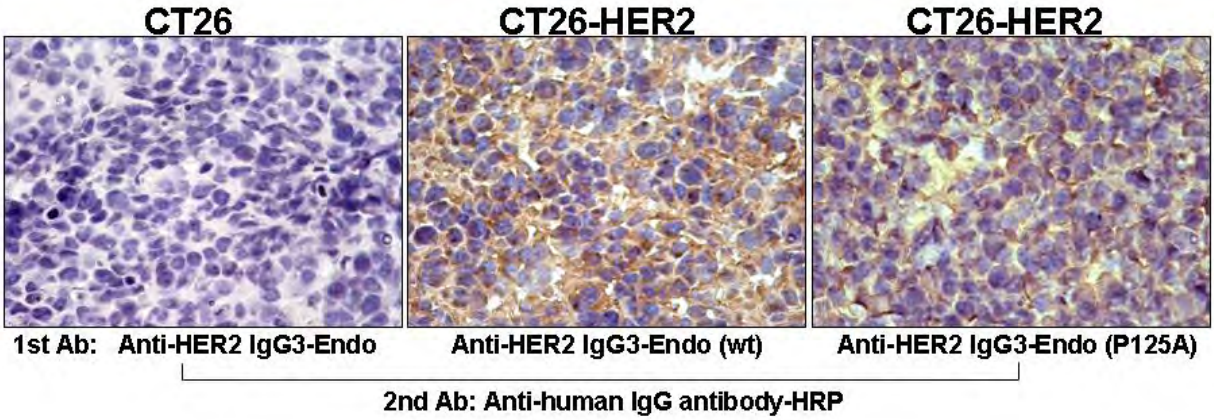


Fig. 8. Targeting of anti-HER2 IgG3-human endostatin fusion proteins to the HER2 antigen on tumors. CT26 and CT26-HER2 tumor sections were incubated with anti-HER2 IgG3-endostatin fusions. Each cryosection was stained with anti-human IgG-HRP and visualized with DAB. All sections were counterstained with hematoxylin.

antigen on CT26-HER tumor, but neither of them recognized CT26 tumor.

Since the endostatin fusion domain may affect folding and possibly its antibody effector functions (ADCC or CDC), Anti-HER2 IgG3-C_H3-Endo (wild type) was compared with the parental anti-HER2 IgG3 antibody and an irrelevant anti-Dansyl IgG3 antibody (Fig. 9). ADCC activity is determined by a ^{51}Cr-release assay.[20,21] CT26-HER and SK-BR-3 were used as target cells and CT26 was used as a negative control target. Anti-HER2 IgG3-C_H3-Endo demonstrated similar ADCC activity of the parental anti-HER2 IgG3 antibody, while the irrelevant antibody shoed no ADCC. The endostatin moiety of the fusion protein did not inhibit antibody effector function.

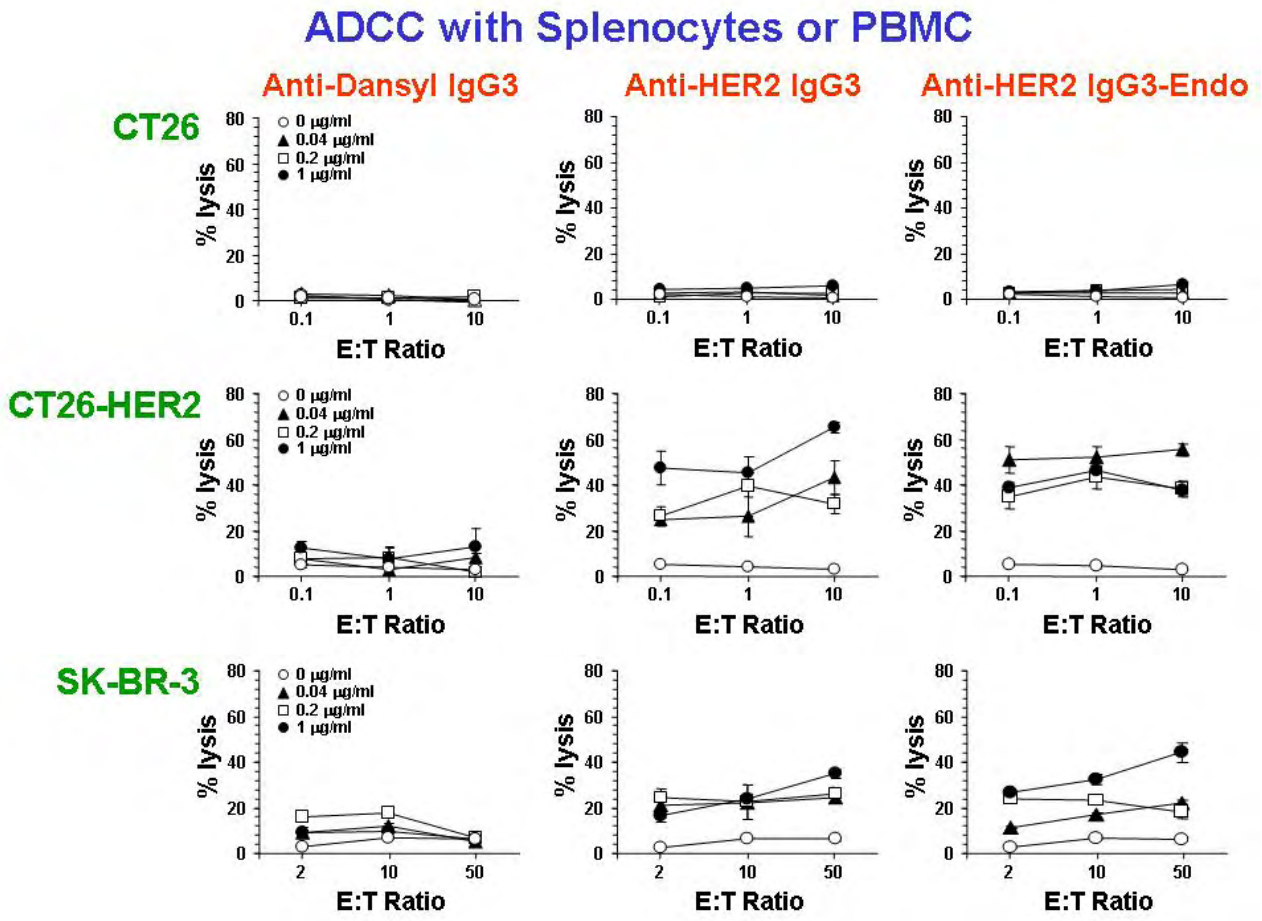


Fig. 9. Antibody-dependent cellular cytotoxicity of CT26-HER2, CT26, or SK-BR-3 cells. ^{51}Cr-labeled target cells were incubated in the presence of various concentrations of antibody fusion proteins plus mouse splenocytes (effector/target ratio: 0.1 to 10) or human PBMC (effector/target ratio: 2 to 50) for 4 hr. Percent specific lysis was calculated as [(experimental release - spontaneous release)/(maximal release - spontaneous release)] × 100. The data were presented as the means of triplicate determinations ± SEM.

Task 3. Determine pharmacokinetics, tumor targeting ability, and tissue biolocalization of endostatin fusion proteins (Months 7-12).

Task 4. Analyze antigenicity of the fusion proteins by ELISA (Months 7-12).

Before we determine pharmacokinetics, biolocalization, or antigenicity of endostatin fusion proteins, we would like to examine the antiangiogenic efficacy and anti-tumor ability of the fusion proteins first. If we have any reduced efficacy of the endostatin fusion proteins, we would revisit these tasks to improve the efficacy of the fusion proteins, but the fusion proteins showed markedly increased anti-tumor efficacy.

<u>Specific Aim II.</u> Test the antiangiogenic activity of anti-HER2 antibody-human endostatin fusion protein(s) *in vitro* and *in vivo*.

***Task5*.** Analyze HER2 signaling (Months 4-18).

<u>Anti-proliferation activity of the endostatin fusion proteins on SK-BR-3 tumor cells</u>

Targeting of anti-HER2 IgG-huEndo fusion proteins to tumors may inhibit HER2 signaling. [^{3}H]-thymidine uptake[22] was measured to determine the effect of anti-HER2 antibody-huEndo fusion proteins on proliferation of human breast cancer cell SK-BR-3 (Fig. 10). Endostatin and endostatin fusion proteins inhibited proliferation of SK-BR-3 in a U-shape manner dependent on concentrations.[23] The concentration of 22.73 nM showed the maximal anti-proliferation activity on SK-BR-3 with endostatin or endostatin fusion proteins. SK-BR-3 proliferation was more effectively inhibited by anti-HER2 IgG3-muEndo (αHER2-muEndo. $p = 0.0512$) than murine endostatin (muEndo) and by anti-HER2 IgG3-huEndo (wt, αHER2-huEndo. $p = 0.0053$) and anti-HER2 IgG3-huEndo (mt: P125A, αHER2-huEndo-P125A. $p = 0.0207$) than human endostatin (huEndo). Endostatin fusion proteins were significantly effective than anti-HER2 IgG3 (αHER2 IgG3), but muEndo ($p = 0.0986$) and huEndo ($p = 0.0932$) were not significantly different.

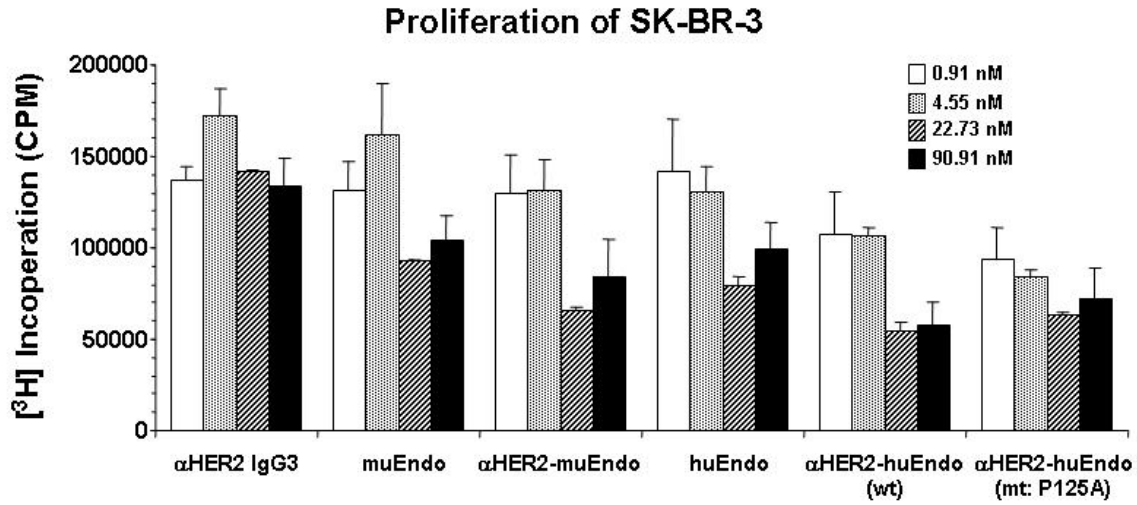


Fig.10. Proliferation of human breast tumor cell, SK-BR-3. SK-BR-3 cells (5000 cells/well) were incubated with endostatin or endostatin fusion proteins at the indicated concentration for 5 days and [^{3}H]-thymidine (1 μCi/well) was added 16 hrs before harvesting. The data were presented as the means of triplicate determinations ± SEM.

***Task 6*.** Analyze antiangiogenic activities (Months 8-18).

<u>Inhibition of endothelial tube formation by the αHER2-huEndo fusion protein</u>

To evaluate potential functions of the human endostatin moiety in the antibody fusion proteins, we explored the function of the αHER2-huEndo fusion proteins in an in vitro morphogenesis assay during which endothelial cells plated on ECM preparations, such as Matrigel, spontaneously aggregate and assemble into densely multicellular capillary-like tubular structures.[24,25] The αHER2-huEndo fusion proteins treatment at the time of HUVEC plating on Matrigel strongly inhibited assembly into tubular structures, with cells remaining dispersed and exhibiting a morphology resembling cells on plastic rather than aggregation into characteristic capillary-like tubes.[26] Tubular structures by light microscopy

revealed a dose-dependent effect (Fig. 11). The αHER2-huEndo fusion proteins showed significant inhibition of HUVEC tube formation compared to αHER2 IgG3 and human endostatin. In addition, αHER2-huEndo-P125A inhibited more effectively than αHER2-huEndo (Fig. 11). These results suggest that a mutation of proline to alanine at amino acid position 125 of human endostatin may have increased anti-tubule forming efficacy of human endostatin.

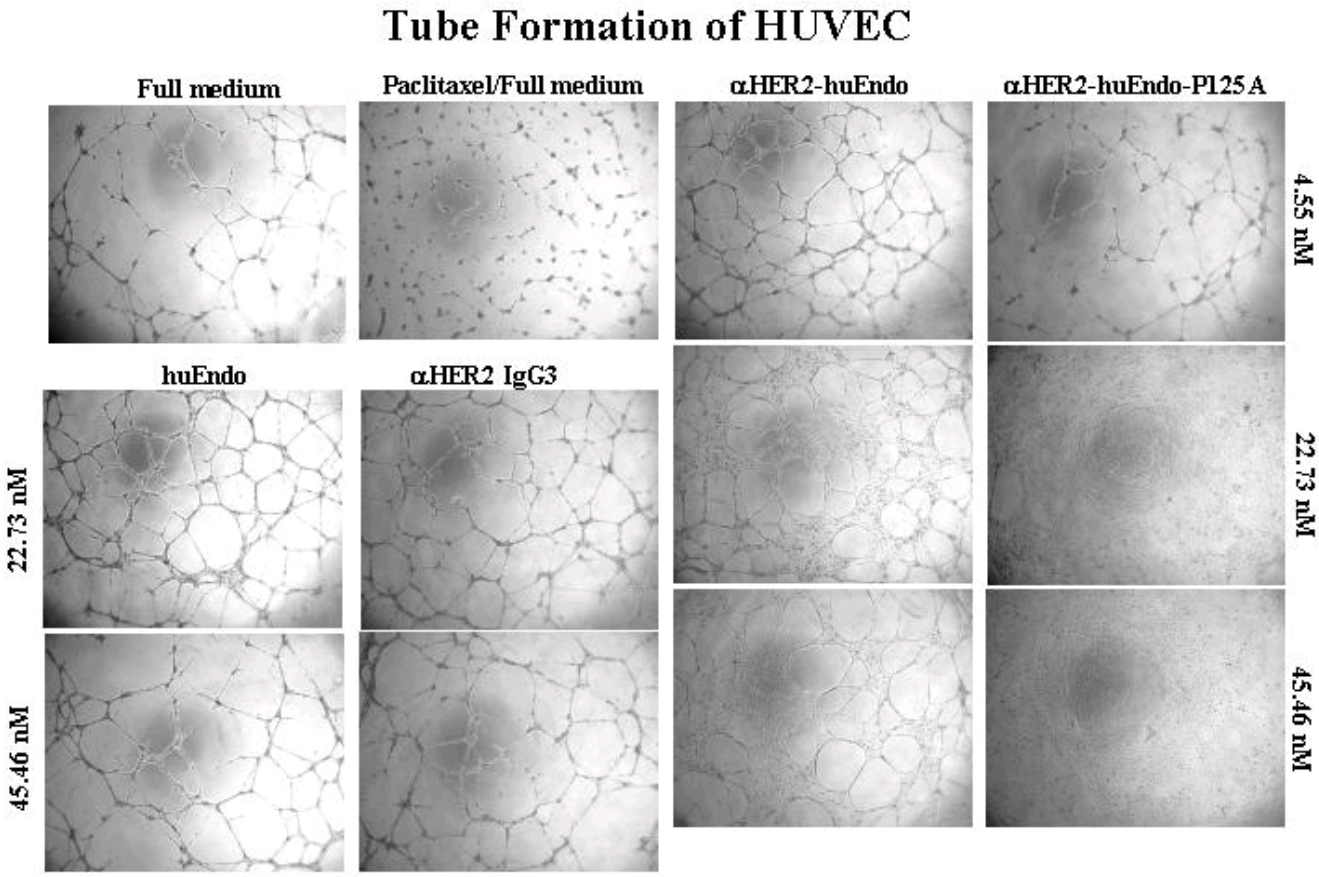


Fig. 11. Effects of anti-HER2 IgG3-huEndo fusion proteins on EC tube formation. HUVECs (4×10^4 cells) were resuspended in 300 μl of full endothelial cell growth medium and treated with the various anti-HER2 IgG3-huEndo fusion proteins before plating onto the Matrigel-coated plates. After 12-16 hr of incubation, tube formation was observed through an inverted photomicroscope. Full media and paclitaxel were used as negative and positive control, respectively. The data were presented as the means of triplicate determinations ± SEM.

Proliferation of endothelial cells by the αHER2-huEndo fusion proteins

The effect of the αHER2-huEndo fusion proteins on endothelial cell (EC) proliferation was assessed. HUVECs were exposed to increasing concentrations of the fusion proteins for 72 hrs in the absence or presence of either VEGF or bFGF as angiogenic stimulus.[24,25] As shown in Fig. 12, both αHER2-huEndo fusion proteins inhibited growth factor-induced proliferation of HUVEC in a concentration-dependent manner. The concentrations necessary for half maximal inhibition (IC50) were 31.3 nM for αHER2-huEndo and 15.2 nM for αHER2-huEndo-P125A in minimal media (1%FBS/EGM-2), 34.2 nM for αHER2-huEndo and 17.8 nM for αHER2-huEndo-P125A in bFGF/1%FBS/EGM-2, or 33.6 nM for αHER2-huEndo and 15.7 nM for αHER2-huEndo-P125A in VEGF/1%FBS/EGM-2. The αHER2-huEndo fusion proteins were also capable of inhibiting the endothelial cell proliferation stimulated by angiogenic factors, VEGF and bFGF (Fig. 12). HUVEC proliferation was more effectively inhibited by αHER2-huEndo-P125A than by αHER2-huEndo.

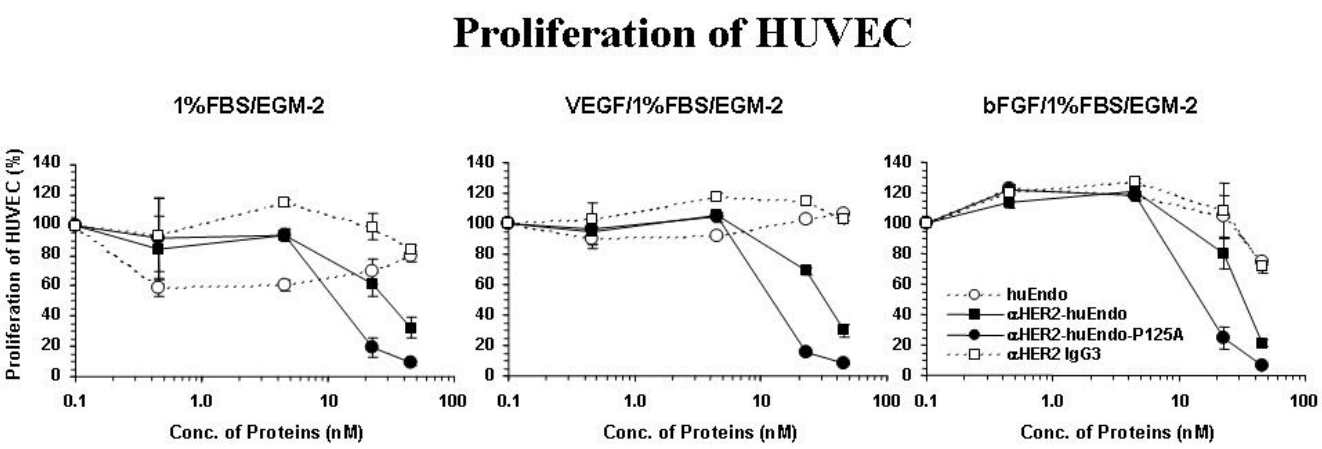


Fig. 12. Effects of anti-HER2 IgG3-huEndo fusion proteins on EC proliferation. HUVECs (4 x 10^3 cells) were treated with increasing concentrations of the endostatin fusion proteins and proliferation was measured at 72 hrs. The data were presented as the means of triplicate determinations ± SEM.

Task 7. Analysis of antiangiogenesis (Months 12-24).
Task 8. Examine VEGF/VEGFR and PDGF/PDGFR expression in tumors (Months 12-24).

Binding ability of anti-HER2 human endostatin fusion proteins to HUVECs and immunofluorescent staining of blood vessel of treated tumor

To determine whether anti-HER2 IgG3-huEndo fusion proteins could bind to endothelial cells, HUVECs were treated with anti-HER2 IgG3, huEndo, or αHER2-huEndo fusion proteins, and human endostatin domain bound to HUVECs was detected with biotinylated anti-human endostatin and stained with streptoavidin-PE conjugate. Binding of human endostatin and αHER2-huEndo fusion proteins to HUVECs was readily detected, while the isotype control, or anti-HER2 IgG3 binding was not detected (Fig. 13A). Of note αHER2-huEndo-P125A showed slightly augmented binding to HUVECs relative to either human endostatin or αHER2-huEndo.

To investigate the effects of αHER2-huEndo-P125A fusion protein on tumor angiogenesis on the EMT6 model, tumors were resected (2 mice per group, control and αHER2-huEndo-P125A treated mice) after 4 or 7 treatments, and tumor microvasculature was visualized using anti-PECAM fluoresecence immunostaining. We stained histologic sections of tumors derived from treated and untreated mice for vasculature using anti-PECAM antibody (Fig. 13B). Immunofluorescent staining of EMT6-HER2 tumors demonstrated that the antibody-endoststin fusion treated group showed thin, short, and fragmented blood vessels on day 12 (4 treatments, Fig. 13B: F-H) compared to those of the PBS treated group (Fig. 13B: B-D). By day 18 (7 treatments), EMT6-HER2 tumor from one of two mice treated with αHER2-huEndo-P125A had completely regressed and the other in the treated group demonstrated very small tumor without stainable vessels, while vasculature was readily demonstrated in the PBS treated (Fig. 13C). No inhibition of vasculature in EMT6-HER2 tumor was seen with anti-HER2 antibody or with human endostatin alone (data not shown).

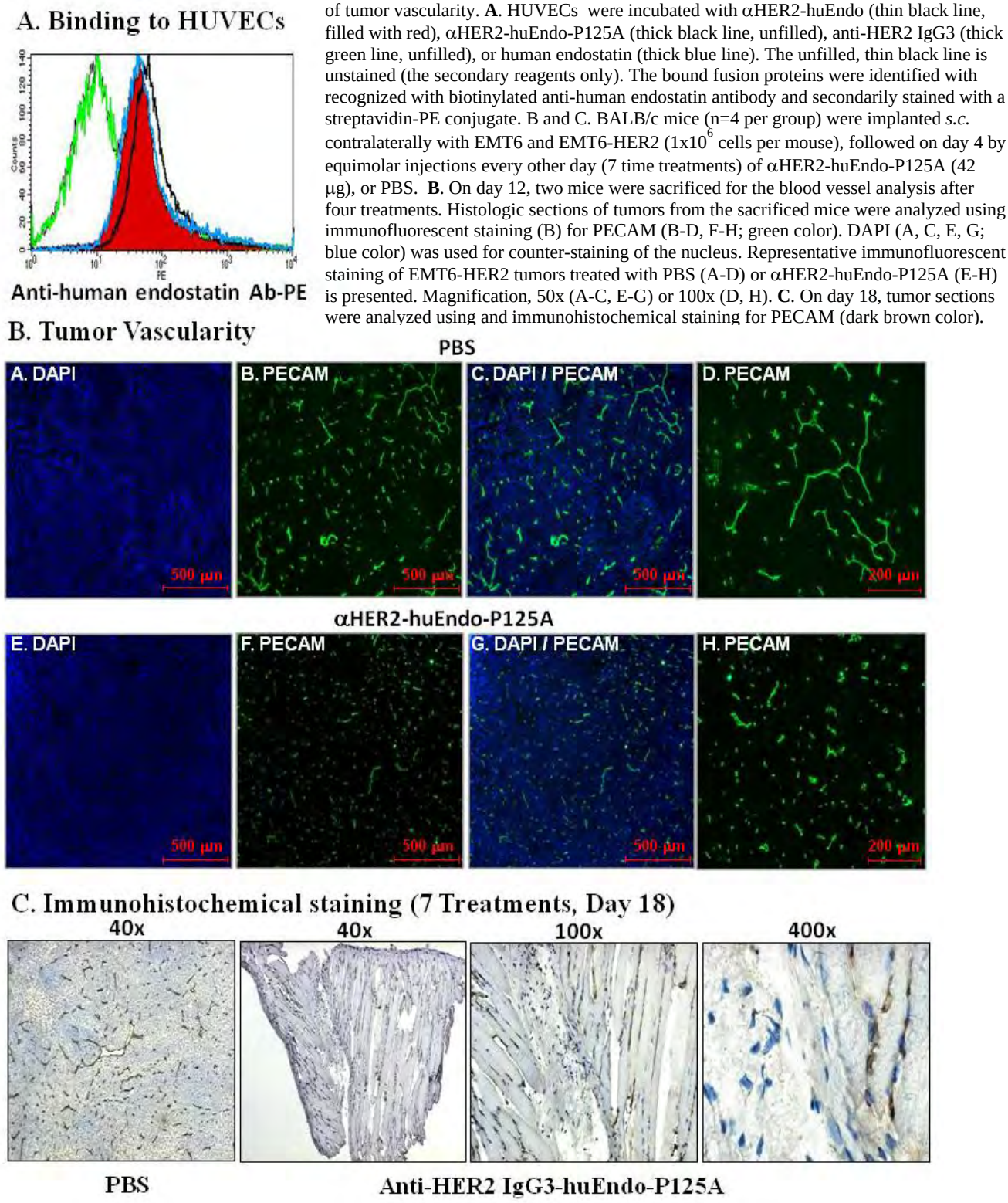


Fig. 13. Binding of anti-HER2 human endostatin fusion proteins to HUVECs, and analysis of tumor vascularity. **A**. HUVECs were incubated with αHER2-huEndo (thin black line, filled with red), αHER2-huEndo-P125A (thick black line, unfilled), anti-HER2 IgG3 (thick green line, unfilled), or human endostatin (thick blue line). The unfilled, thin black line is unstained (the secondary reagents only). The bound fusion proteins were identified with recognized with biotinylated anti-human endostatin antibody and secondarily stained with a streptavidin-PE conjugate. B and C. BALB/c mice (n=4 per group) were implanted *s.c.* contralaterally with EMT6 and EMT6-HER2 ($1x10^6$ cells per mouse), followed on day 4 by equimolar injections every other day (7 time treatments) of αHER2-huEndo-P125A (42 μg), or PBS. **B**. On day 12, two mice were sacrificed for the blood vessel analysis after four treatments. Histologic sections of tumors from the sacrificed mice were analyzed using immunofluorescent staining (B) for PECAM (B-D, F-H; green color). DAPI (A, C, E, G; blue color) was used for counter-staining of the nucleus. Representative immunofluorescent staining of EMT6-HER2 tumors treated with PBS (A-D) or αHER2-huEndo-P125A (E-H) is presented. Magnification, 50x (A-C, E-G) or 100x (D, H). **C**. On day 18, tumor sections were analyzed using and immunohistochemical staining for PECAM (dark brown color).

<u>Specific Aim III.</u> Study the antibody-endostatin fusion proteins *in vivo* for effects on tumor growth in animal tumor and/or human xenograft models.

Task 9. Anti-tumor activity in human tumor xenografts (Months 18-24)

<u>Anti-tumor efficacy in human breast cancer SK-BR-3 xenografts</u>

Herceptin, anti-HER2 IgG1, is able to inhibit the growth of human breast cancer SK-BR-3 overexpressing HER2. SK-BR-3 may need both signaling through HER2 and neoangiogenesis for optimal growth. We assayed for anti-tumor activity of α HER2-huEndo fusion proteins against human breast cancer SK-BR-3 in SCID mice.[9] Equimolar proteins were injected every other day. The SK-BR-3 tumor xenografts showed the greatest differences among the treatments (Fig. 14). In Fig. 14A, huEndo and αHER2 IgG3 inhibited 48.36% (*p* value = 0.0123) and 55.46% (*p* value = 0.0046) of tumor growth relative to the non-treated group (PBS) on day 29, while αHER2-huEndo and αHER2-huEndo-P125A inhibited 82.58% (*p* value = 0.0111) and 98.66 % (*p* value = 0.0090), respectively. It is noteworthy that the treatment with αHER2-huEndo-P125A completely eradicated tumors after 30 days. The proportion of tumor-free survivors was higher for the αHER2-huEndo-P125A group (5 of 5) compared to PBS (0 of 5), αHER2 IgG3 and huEndo (1 of 5), and αHER2-

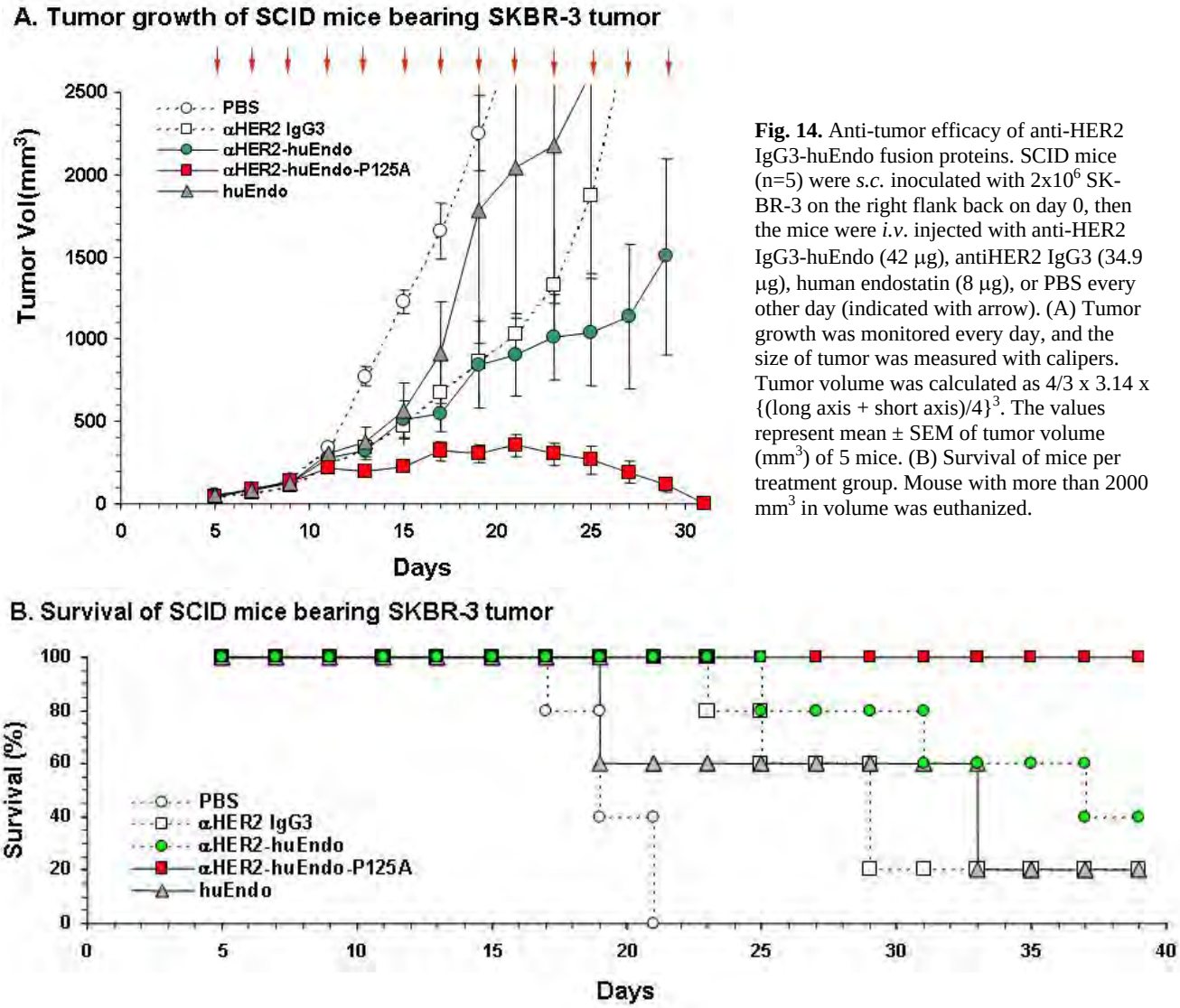


Fig. 14. Anti-tumor efficacy of anti-HER2 IgG3-huEndo fusion proteins. SCID mice (n=5) were *s.c.* inoculated with $2x10^6$ SK-BR-3 on the right flank back on day 0, then the mice were *i.v.* injected with anti-HER2 IgG3-huEndo (42 μg), antiHER2 IgG3 (34.9 μg), human endostatin (8 μg), or PBS every other day (indicated with arrow). (A) Tumor growth was monitored every day, and the size of tumor was measured with calipers. Tumor volume was calculated as 4/3 x 3.14 x {(long axis + short axis)/4}^{3}. The values represent mean ± SEM of tumor volume (mm^3) of 5 mice. (B) Survival of mice per treatment group. Mouse with more than 2000 mm^3 in volume was euthanized.

huEndo (2 of 5) (Fig.14B). αHER2-huEndo-P125A showed a greater extent survival rate of the human breast cancer SK-BR-3 xenografts in SCID mice than αHER2 IgG3, huEndo, or αHER2-huEndo.

Anti-tumor efficacy in murine mammary tumor EMT6 model

In previous study, we demonstrated that the *in vivo* targeting of antiangiogenic proteins using αHER2-murine Endo fusion protein enhanced preferential inhibition of tumor growth expressing HER2, compared to contralaterally implanted parental tumor (no HER2 expression). To investigate whether the ability of αHER2-huEndo fusion proteins to specifically target tumors bearing HER2 may

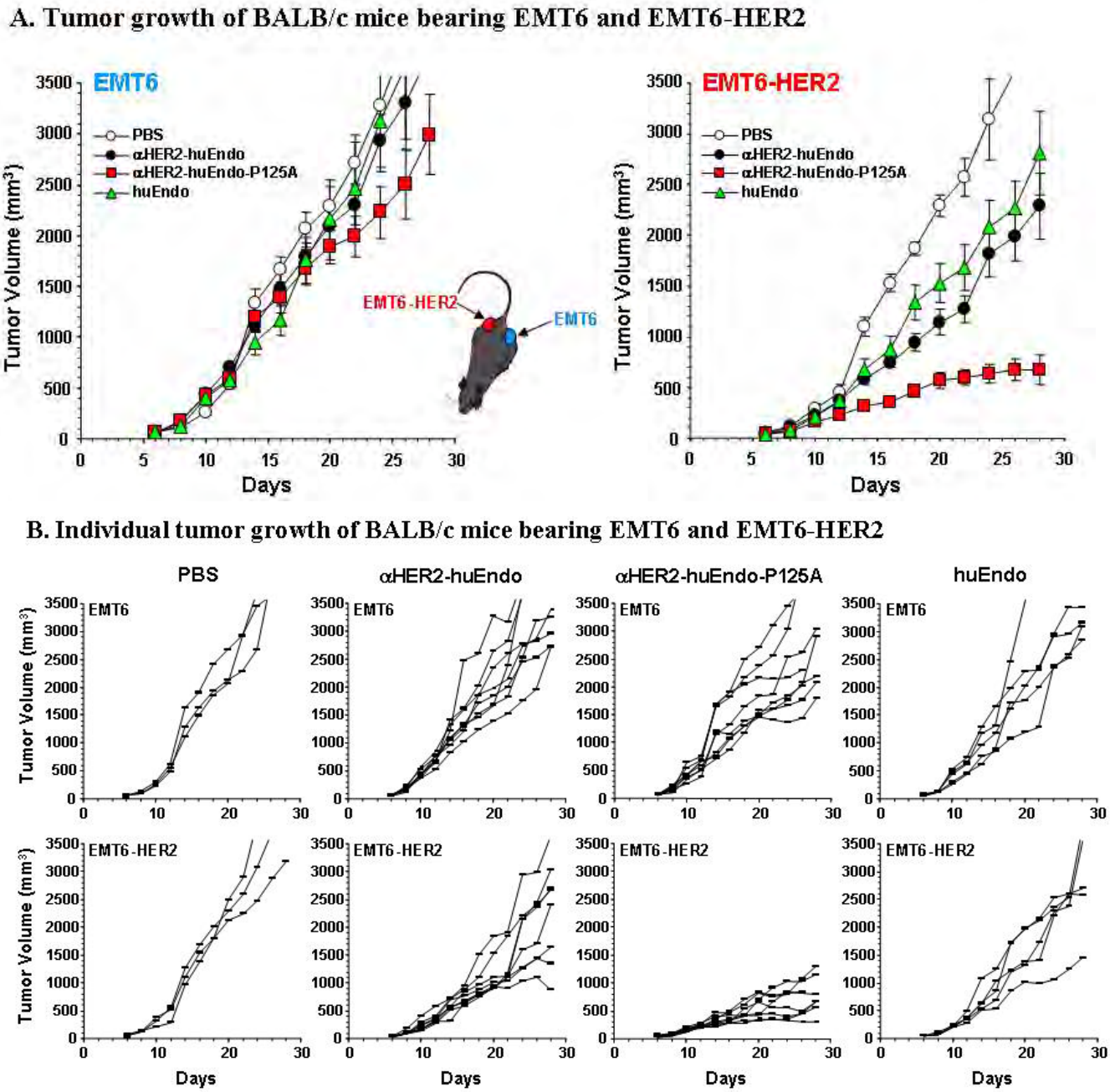


Fig. 15. Anti-tumor activity of αHER2-huEndo fusion proteins in a syngeneic mouse model. A. Tumor growth of mice bearing murine mammary tumor EMT6 and EMT6-HER2. BALB/c mice (n=3-8 per group) were implanted *s.c.* contralaterally with EMT6 and EMT6-HER2 ($1x10^6$ cells per mouse), followed on day 6 by equimolar injections every other day (11 times) of anti-HER2 IgG3-huEndo (42 μg), human endostatin (8 μg), or PBS. Tumor measurements are described above and are presented as mean ± SEM. B. Individual tumor growth of mice treated with anti-HER2-huEndo fusion proteins.

increase efficacy, BALB/c mice simultaneously implanted with mouse mammary EMT6 and EMT6-HER2 tumors on opposite flanks were treated with either α HER2-huEndo-P125A, α HER2-huEndo, or huEndo.9 Equimolar administration of αHER2-huEndo-P125A to mice showed preferential growth inhibition of EMT6-HER2, compared to EMT6 parental tumor implanted on the contralateral flank (Fig. 15). The ability of αHER2-huEndo fusion proteins to specifically target tumors bearing HER2 increased anti-tumor efficacy. αHER2-huEndo-P125A inhibited EMT6-HER2 tumor growth more effectively than PBS (*p* value = 0.0025), huEndo (*p* value = 0.0031), or αHER2-huEndo (*p* value = 0.0041). The mutation of human endostatin moiety of αHER2-huEndo-P125A fusion protein therefore increased the potential efficacy of current antiangiogenic strategies of αHER2-huEndo fusion proteins.

Task 10. Anti-tumor activity in metastatic models (Months 21-27)
Task 11 (old Task 12: Mislabeled). Anti-tumor activity in orthotopic metastatic models (Months 24-36)

We have already developed another murine mammary tumor model, 4T1 and 4T1-HER2, which is well known as a murine orthotopic breast metastatic tumor (Fig. 16). We now continue to test anti-tumor efficacy of the fusion proteins in an orthotopic animal model with 4T1 and 4T1-HER2.

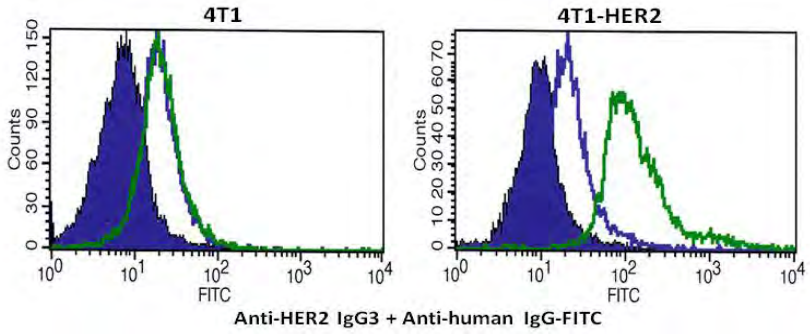


Fig. 16. HER2 expression of 4T1. Murine mammary tumor cells, 4T1 and 4T1-HER2, were incubated with αHER2IgG3 and the bound αHER2 human IgG3 was identified with anti-human IgG-FITC conjugated (thick green line). The unfilled, thick blue line is stained with the secondary reagent (anti-human IgG-FITC) only, and the blue filled histogram is unstained.

Task 12 (old Task 13: Mislabeled). Combination treatment (Months 24-36)
PDGF blockade: Imatinib (Months 24-30)
VEGF blockade: Avastin (Months 24-30)
Metronomic therapy (Months 27-36)

We started the combination therapy with anti-HER2 IgG3-huEndo (wild type) protein only, because anti-HER2 IgG3-huEndo-P125A (mutant type) alone was able to show great enhanced anti-tumor activity.

PDGF blockade: Imatinib (Gleevec)

Gleevec (STI57, imatinib, Novartis Pharma AG) has been approved for chronic myelogenous leukemia and gastrointestinal stromal tumors. Gleevec disrupts the association of pericytes with neovasculature in tumors through its effects on PDGFR.[27] While endostatin inhibits early blood vessel formation, imatinib may affect maturation by acting on pericytes.[27,28] We initially treated EMT6 and EMT6-HER2 tumors, subcutaneously implanted on the left and right flank respectively, with combination of anti-HER2-huEndo fusion protein and gleevec.[9] However, EMT6 and EMT6-HER2 tumors were very sensitive to gleevec, and no additive or synergistic effect was observed. We repeated the gleevec combination therapy on the CT26 and CT26-HER2 tumor model (Fig. 17). Although

CT26/CT26-HER2 tumors were less sensitive to gleevec than EMT6/EMT6-HER2, the combination of αHER2-huEndo and gleevec did not show significantly increased anti-tumor efficacy compared to αHER2-huEndo (p = 0.118) or gleevec alone (p = 0.070).

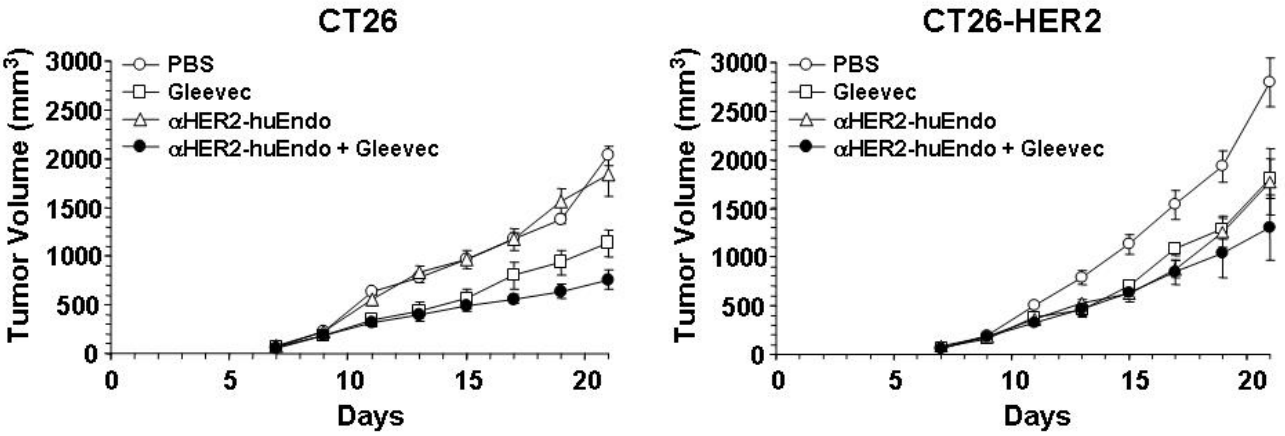


Fig. 17. Evaluation of the combination therapeutic effect with gleevec on established tumor. BALB/c mice (n=5) were implanted *s.c.* contralaterally with CT26 and CT26-HER2 ($1x10^6$ cells per mouse), followed on day 7 by every other day of αHER2-huEndo (42 μg), gleevec (*p.o.* 2 mg/day), or combination of αHER2-huEndo and gleevec. Tumor measurements are described above and are presented as mean ± SEM.

VEGF Blockade: Bevacizumab (Avastin)

A humanized anti-VEGF antibody (bevacizumab, Avastin™, rhuMAb-VEGF; Genentech) binds and neutralizes all of the major isoforms of VEGF-A, decreasing vascular volume, microvascular density, interstitial fluid pressure and the number of viable, circulating endothelial cells.[29] Therefore, combining fusion proteins with Avastin may augment activity of both approaches. We studied whether αHER2-huEndo, Avastin, αHER2 IgG3, or both αHER2-huEndo and Avastin in combination would

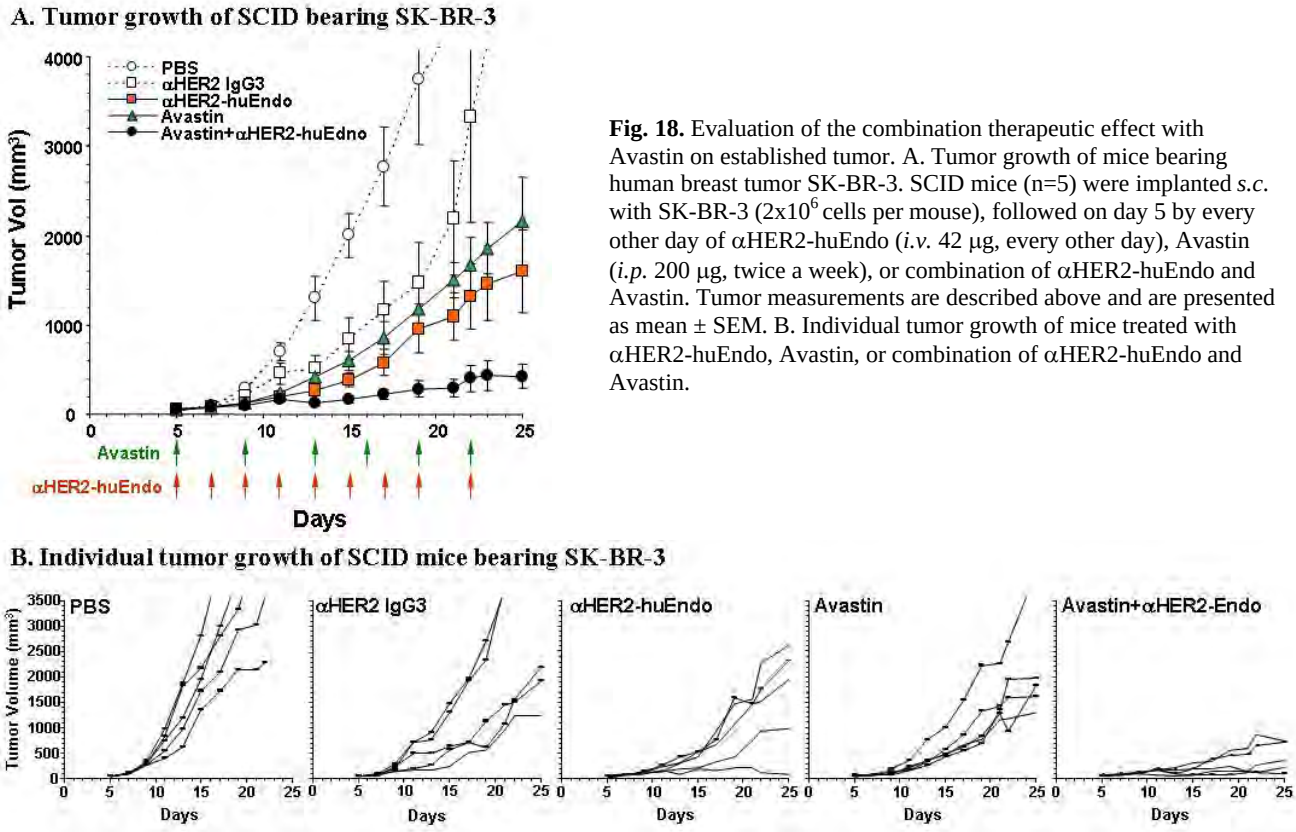


Fig. 18. Evaluation of the combination therapeutic effect with Avastin on established tumor. A. Tumor growth of mice bearing human breast tumor SK-BR-3. SCID mice (n=5) were implanted *s.c.* with SK-BR-3 ($2x10^6$ cells per mouse), followed on day 5 by every other day of αHER2-huEndo (*i.v.* 42 μg, every other day), Avastin (*i.p.* 200 μg, twice a week), or combination of αHER2-huEndo and Avastin. Tumor measurements are described above and are presented as mean ± SEM. B. Individual tumor growth of mice treated with αHER2-huEndo, Avastin, or combination of αHER2-huEndo and Avastin.

inhibit the growth of human breast cancer SK-BR-3 xenografts in SCID mice. SK-BR-3 was implanted on the flank of SCID mice.[9] The treatment was repeated (shown below in Fig. 18). Administration of αHER2-huEndo or Avastin alone showed enhanced anti-tumor activity, compared to either αHER2 IgG3 alone (p=0.0208, 0.0387 respectively), or the untreated group (p=0.0135, 0.0150 respectively). As expected, αHER2-huEndo and Avastin given in combination resulted in a significantly and synergistically greater reduction of tumor volume, compared to either αHER2-huEndo (p=0.0051), Avastin (p=0.0036), αHER2 IgG3 (p=0.0149), or the untreated group (p=0.0141) (Fig. 18).

KEY RESEARCH ACCOMPLISHMENTS

- Treatment of established SK-BR-3 xenografts in SCID mice with the αHER2-huEndo-P125A fusion resulted in greater inhibition of growth, compared to αHER2 IgG3, human endostatin, or αHER2-huEndo fusion protein treated mice. The αHER2-huEndo fusion protein specifically inhibited HER2+ tumors in syngeneic mice simultaneously implanted with EMT6 and EMT6-HER2.

- The αHER2-huEndo and αHER2-huEndo-P125A fusion proteins markedly inhibited endothelial tube formation and proliferation of HUVEC *in vitro*, and did so more efficiently than human endostatin. The αHER2-huEndo-P125A fusion protein showed greater inhibition of tube formation *in vitro* than either native endostatin or than wild type αHER2-huEndo fusion. Since the αHER2-huEndo fusion proteins retain two endostatin domains in a fusion protein, they may effectively present endostatin as a dimer, and this may result in enhanced anti-angiogenic activity.

- Combining the targeting capability of anti-HER2 antibody with the anti-angiogenic activity of human endostatin presented in a dimer form in the context of a fusion antibody improves the inhibition of endothelial tube formation and proliferation of HUVEC *in vitro* and enhances anti-tumor activity *in vivo*.

REPORTABLE OUTCOMES

U.S. provisional patent application

A provisional patent application (60/946,245) has been filed on June 26, 2007 (Appendix 1 and Appendix 2). A regular U.S. patent application will be filed before June 28, 2008.

Manuscript submission

A manuscript entitled, "Targeted delivery of anti-HER2 antibody-human endostatin P125A mutant fusion protein results in enhanced anti-tumor efficacy in murine and human breast tumor models", has been submitted for publication in Cancer Research (Appendix 3 and Appendix 4).

Poster presentation

A poster entitled, "Targeted delivery of anti-HER2 antibody-human endostatin P125A mutant fusion protein results in enhanced anti-tumor efficacy in murine and human breast tumor models", has been presented at the 99th Annual Meeting of the American Association of Cancer Research, 2008 Apr 12-16, San Diego, CA, Abstract #1105 (Appendix 5 and Appendix 6)

CONCLUSIONS

Linking human endostatin to an antibody may significantly enhance anti-tumor activity of trastuzumab. Mutant P125A fusion antibody showed much better anti-tumor activity. Combination with other anti-angiogenic drugs synergistically augmented anti-tumor efficacy. Targeting anti-angiogenic proteins using antibody is a versatile approach that could be applied to other targets (*e.g.* EGFR, PSMA), or using other anti-angiogenic protein domains.

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APPENDICES

Appendix 1: US Provisional Patent Application (60/946,245; 06/26/2007)
Appendix 2: UM08-01 Antibody-endostatin fusion protein and its variants
Appendix 3: Manuscript submission letter
Appendix 4: Manuscript
Appendix 5: AACR Poster acceptance letter
Appendix 6: AACR Poster

SUPPORTING DATA

Not applicable.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO
60/946,245	06/26/2007		100	7230-38

CONFIRMATION NO. 3201

30448
AKERMAN SENTERFITT
P.O. BOX 3188
WEST PALM BEACH, FL33402-3188

FILING RECEIPT

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Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

SEUNG-UON SHIN, Miami, FL;
Joseph D. Rosenblatt, Hollywood, FL;
Sherie L. Morrison, Los Angeles, CA;

Power of Attorney: The patent practitioners associated with Customer Number 30448

If Required, Foreign Filing License Granted: 07/10/2007

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US60/946,245**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

** SMALL ENTITY **

Title

ANTIBODY-ENDOSTATIN FUSION PROTEIN AND ITS VARIANTS

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Medical Category: Drugs

Medical Specialty: Cancer and Immunology

Engineering or Physical Sciences Category:

ID Number: UM08-01

Contact email: Efenjves@miami.edu

Brief

Technology: Antibody-endostatin fusion protein and its variants.

Scientific Relevance: This invention relates to compositions and methods for targeting and modulating the activity of tumor cells. In particular, the invention relates to chimeric fusion molecules which have a tumor antigen targeting domain and an anti-tumor effector function domain.

Commercial Opportunity: The sale of anti angiogenic molecules have reached the $600million level and it is predicted by Forbes magazine to reach the billion dollar level in the next few years when it becomes applicable to colon, bladder, head and neck and prostate cancer. This approach is a huge improvement over these drugs.

Competitive Advantage: As effective as anti angiogenic molecules are they have half life and concentration problems that this technology addresses.

Inventors: Sherrie Morrison, Joseph D. Rosenblatt, and Seung-uon Shin

More Details

Antibody-human endostatin fusion protein and its variants (UM08-01)

Drs. Sherrie Morrison (UCLA), Joseph D. Rosenblatt, and Seung-uon Shin

PROBLEM
Our ability to specifically target and modulate the activity of tumor cells is limited by the half life of targeting molecules. Anti-angiogenic tumor therapies have recently attracted intense interest because of their broad-spectrum action, low toxicity, and absence of drug resistance. However if these molecules could have a tumor antigen targeting domain and an anti-tumor effector function domain this would be an ideal addition to the armamentarium available for cancer.

SOLUTION
Endostatin is a well characterized anti-angiogenic agent which has a short half life. The invention provides methods and compositions for targeting a chimeric molecule containing both (1) anti-angiogenic agent and (2) a carrier domain such as all or a portion of an Ig molecule to a tumor making it more specific and longer lived.

APPLICATIONS
The inventors have developed a novel approach to create a human fusion protein and have used it to construct endostatin fused to targeting sequences derived from an anti HER 2 antibody.

- CONSTRUCTION OF SEVERAL ANTI-HER2 IGG3-HUMAN ENDOSTATIN FUSION PROTEINS BY FUSING HUMAN ENDOSTATIN OR ENDOSTATIN DERIVATIVES TO THE 3' END OF A HUMANIZED ANTI-HER2 IGG3 ANTIBODY FOR THE PURPOSE OF:
    - ENHANCING LOCAL DELIVERY OF ENDOSTATIN TO TUMOR
    - INCREASING ENDOSTATIN HALF-LIFE.
    - TARGETING ENDOSTATIN TO THE HER2 TUMOR
    - ACHIEVING HIGHER LOCAL CONCENTRATION OF ENDOSTATIN
    - INCREASING ENDOSTATIN ACTIVITY THROUGH PRESENTATION AS A DIMER

PATENT STATUS
United States Provisional Patent Appln No. 60/945,245 entitled "ANTIBODY-ENDOSTATIN FUSION PROTEIN AND ITS VARIANTS" filed on June 26, 2007.

Competitive Advantage
Anti angiogenic molecules have a very short half life and have to be administered at very high concentrations. Both of these issues are addressed with the current technology.

LICENSING OPPORTUNITY
Seeking collaborative research and licensing options.

About the Inventors

Joseph D. Rosenblatt, M.D.
Joseph D. Rosenblatt, M.D., is the William Harrington Professor of Medicine, Microbiology and Immunology and Chief of the Division of Hematology/Oncology in the Department of Medicine at the University of Miami/Miller School of Medicine and also serves in the role of the Associate Director for Clinical and Translational Research of the University of Miami Sylvester Comprehensive Cancer Center. Dr. Rosenblatt is well known in the area of cancer immunotherapy and gene therapy, and has been funded by the National Cancer Institute and the Department of Defense to pursue development of novel antibody fusion proteins, along with his co-inventor, Dr. Seung-Uon Shin. Dr. Rosenblatt is an authority on the use of co-stimulatory ligands, and other immune effector molecules delivered through gene transfer and/or antibody fusion proteins for purposes of augmenting immune responses to human and murine tumors.

Dr. Seung-Uon Shin, M.D.
Dr. Seung-Uon Shin, M.D., is a Research Associate Professor of Clinical Medicine in the Division of Hematology/Oncology at the University of Miami/Miller School of Medicine. He received his Ph.D. in Cell Biology in 1987 from the Albert Einstein College of Medicine and also holds an M.S. in Pharmacy from the Seoul National University. Dr. Shin is a recognized authority in the area of antibody fusion protein production, having performed his postdoctoral research in the laboratory of Dr. Sherie Morrison, one of the world's premier antibody engineering laboratories. Drs. Rosenblatt and Shin have co-authored numerous articles together on the use of antibody fusion proteins as agents directed against breast and other cancers.

Dr. Sherrie Morrison, M.D.
Dr. Sherrie Morrison, M.D. is a Distinguished Professor in the Department of Microbiology, Immunology and Molecular Genetics at the University of California in Los Angeles. From 1992-2002 she served as department chair. Prior to coming to UCLA Dr. Morrison was a Professor in the Department of Microbiology at Columbia University, College of Physicians and Surgeons. She received her B.A. and Ph.D. from Stanford University and completed post-doctoral training at Columbia University, Albert Einstein College of Medicine and University of California at Berkeley. She has published over 230 articles and was a pioneer in the development of recombinant antibodies and antibody-fusion proteins.

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Corresponding Author	Dr. Seung-Uon Shin (University of Miami School of Medicine)
Contributing Authors	Dr. Hyun-Mi Cho , Dr. Jaime Merchan , Ms. Jin Zhang , Dr. Joseph Rosenblatt
Abstract	The anti-angiogenic protein endostatin demonstrated considerable anti-tumor activity in animal models. However, limited anti-tumor activity has been observed in human Phase I/II trials. Trastuzumab has activity in HER2+ breast cancer used alone or in combination with chemotherapy. Prior studies using an anti-HER2 antibody-murine endostatin fusion demonstrated enhanced anti-tumor activity compared to anti-HER2 antibody or endostatin given alone or in combination. We generated two anti-HER2 human endostatin fusion proteins by fusing human wild type or a mutant form of human endostatin (huEndo-P125A) to the 3' end of a humanized anti-HER2 IgG3 antibody. HuEndo-P125A antibody fusion protein (αHER2-huEndo-P125A) inhibited VEGF and bFGF induced endothelial cell proliferation, and capillary formation in vitro, to a greater degree than wild type endostatin fusion protein (αHER2-huEndo), endostatin alone, or anti-HER2 antibody (αHER2 IgG3). Treatment of SKBR-3 breast cancer xenografts with anti-HER2 IgG3-huEndo-P125A fusion resulted in complete regression, and improved survival, compared to either αHER2 IgG3, human endostatin, or anti-HER2 IgG3-huEndo treated mice. αHER2-huEndo fusion proteins specifically targeted tumors expressing HER2 in mice simultaneously implanted with murine mammary tumor cell line EMT6 and EMT6 engineered to express HER2 antigen (EMT6-HER2). αHER2-huEndo-P125A fusion antibody showed enhanced anti-angiogenic and anti-tumor activity and inhibited EMT6-HER2 growth more effectively than huEndo (p = 0.003), or αHER2-huEndo (p = 0.004). Targeting anti-angiogenic proteins using antibody fusion proteins could improve clinical activity of anti-HER2 antibody and endostatin alike, and provides a versatile approach that could be applied to other tumor targets with alternative antibody specificities or using other anti-angiogenic domains.
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Conflict of Interest

Targeted delivery of anti-HER2 antibody-human endostatin P125A protein results in enhanced anti-tumor efficacy in murine and human breast tumor models

Seung-Uon Shin[1,2], Hyun-Mi Cho[1], Jaime Merchan[1], Jin Zhang[1], and Joseph D. Rosenblatt.

[1]Department of Medicine, Hematology-Oncology, University of Miami School of Medicine and Sylvester Comprehensive Cancer Center, 1475 N.W. 12th Ave. Miami, FL 33136, USA,

[2]**Corresponding author: Seung-Uon Shin, Ph.D.** Department of Medicine, Hematology-Oncology, University of Miami School of Medicine and Sylvester Comprehensive Cancer Center, 1475 N.W. 12th Ave. (D8-4) Miami, FL 33136, USA.

Telephone: (305)243-3668

Fax: (305)243-4787

e-mail: sshin@med.miami.edu

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Key Word

Endostatin, Antibody fusion protein, Angiogenesis, Immunotherapy, HER2

Abbreviation

VEGF: vascular endothelial growth factor

bFGF: basic fibroblast growth factor

EBM-2: Endothelial basal medium 2

HUVEC: Human umbilical vein endothelial cell

Abstract

The anti-angiogenic protein endostatin demonstrated considerable anti-tumor activity in animal models. However, limited anti-tumor activity has been observed in human Phase I/II trials. Trastuzumab has activity in HER2+ breast cancer used alone or in combination with chemotherapy. Prior studies using an anti-HER2 antibody-murine endostatin fusion demonstrated enhanced anti-tumor activity compared to anti-HER2 antibody or endostatin given alone or in combination.

We generated two anti-HER2 human endostatin fusion proteins by fusing human wild type or a mutant form of human endostatin (huEndo-P125A) to the 3' end of a humanized anti-HER2 IgG3 antibody. HuEndo-P125A antibody fusion protein (αHER2-huEndo-P125A) inhibited VEGF and bFGF induced endothelial cell proliferation, and capillary formation *in vitro*, to a greater degree than wild type endostatin fusion protein (αHER2-huEndo), endostatin alone, or anti-HER2 antibody (αHER2 IgG3). Treatment of SKBR-3 breast cancer xenografts with anti-HER2 IgG3-huEndo-P125A fusion resulted in complete regression, and improved survival, compared to either αHER2 IgG3, human endostatin, or anti-HER2 IgG3-huEndo treated mice. αHER2-huEndo fusion proteins specifically targeted tumors expressing HER2 in mice simultaneously implanted with murine mammary tumor cell line EMT6 and EMT6 engineered to express HER2 antigen (EMT6-HER2). αHER2 huEndo-P125A fusion antibody showed enhanced anti-angiogenic and anti-tumor activity and inhibited EMT6-HER2 growth more effectively than huEndo ($p = 0.003$), or αHER2-huEndo ($p = 0.004$). Targeting anti-angiogenic proteins using antibody fusion proteins could improve clinical activity of anti-HER2 antibody and endostatin alike, and provides a versatile approach that could be applied to other tumor

targets with alternative antibody specificities or using other anti-angiogenic domains.

Introduction

Endostatin, a fragment of collagen XVIII, was isolated from mice bearing a haemangioendothelioma (EOMA) and is a specific inhibitor of endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (1). The mechanism of action of endostatin is not fully understood. Systemic therapy with murine endostatin (mEndo) inhibited the growth of Lewis lung carcinomas, fibrosarcomas (T241), melanomas (B16F10), and EOMA, limiting tumor growth to dormant microscopic lesions which do not kill the host (1, 2). Endostatin also suppressed growth of human renal cell cancer in a xenograft model (3). Human and mouse collagen XVIII chains show a high degree of homology (86% identity and >90% similarity in protein sequences) (4). Human endostatin (huEndo) inhibited the growth of several different tumors *in vivo*, such as human glioblastoma (U-87MG), C6 rat glioma, or rat gliosarcomas (BT4Cn) (5-7). Repeated treatment with endostatin led to permanent eradication of tumor in several rodent models (1, 2, 8).

In contrast to preclinical studies, clinical experience with endostatin has been disappointing (9-13). In early human Phase I trials, huEndo administration at variable dose levels and schedules was feasible and safe. However, no consistent evidence for anti-tumor activity or biological activity was demonstrated (9-12). In a Phase II study in forty-two patients with advanced pancreatic neuroendocrine tumors or carcinoid tumors treated with huEndo administered as a twice a day subcutaneous injection, huEndo was associated with minimal toxicity (13). However, no patient achieved a partial response and only two patients had a biochemical response. Therefore, although initial clinical trials proved that endostatin is a very

safe drug delivered in a variety of dose schedules, they did not demonstrate comparable anti-tumor activity compared to that seen in murine models.

Several explanations have been advanced for the failure to see anti-tumor activity in the human setting. It has been suggested that initial preparations of endostatin had suboptimal biologic activity and modified, more potent proprietary formulations (*e.g.* EndoStar) are currently undergoing clinical testing in China (14-16). Moreover, it has been reported that endostatin may be less potent in monomeric form than when delivered as a dimer or trimer (17, 18). Finally a mutant form of endostatin (P125A) has been reported to have enhanced anti-angiogenic activity compared to its wild type counterpart (19-21). Endostatin has also been reported to demonstrate a bimodal response curve such that optimal concentrations may not have been achieved in Phase I/II studies (22).

We hypothesized that several of the logistical disadvantages of the long-term treatment with high dosages of endostatin could be overcome if the half-life of endostatin could be extended, if endostatin could be delivered in dimeric form and if endostatin could be specifically targeted to the tumor, to achieve higher local concentrations and greater specificity.

We have previously reported enhanced anti-tumor activity of an anti-HER2 IgG3-C_H3-mEndo fusion protein formed by joining murine endostatin (mEndo) to the 3' end of a humanized αHER2 IgG3 (23). Our prototypic anti-HER2 IgG3-C_H3-mEndo fusion protein had anti-angiogenic activity, prolonged serum half-life compared to endostatin, targeted HER2 expressing tumors, inhibited *in vivo* tumor growth and provided initial validation for this concept. In this study, we report on the biological activity of an αHER2 IgG3 fused to both wild type and a mutant form (P125A) of human endostatin. We demonstrate that this fusion protein is associated with significantly enhanced *in vitro* and *in vivo* anti-angiogenic and anti-tumor effects in several

cancer models.

Materials and Methods

Cell Lines and Animals

To produce a murine breast tumor expressing human HER2, the murine mammary tumor cell line EMT6 was transduced by use of a retroviral construct containing the cDNA encoding the human HER2 gene (EMT6-HER2). EMT6, EMT6-HER2, the human breast cancer cell line SK-BR-3, and transfected Sp2/0 or P3X63Ag8.653 cells were cultured in Iscove's modified Dulbecco's medium with 5% calf serum.

Human umbilical vein endothelial cells (HUVEC), were obtained from Clontech Lab, Inc. (Palo Alto, CA) and used between passages 3 and 5 and maintained in EGM2-MV medium (Clontech, Palo Alto, CA) that contained endothelial basal medium 2 (EBM-2), supplemented with 5% fetal bovine serum (FBS), gentamicin, amphotericin B, hydrocortisone, ascorbic acid, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), human epidermal growth factor, and insulin-like growth factor I.

Female BALB/c mice (4–6 weeks) and severe combined immunodeficient (SCID) mice (4–6 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for *in vivo* tumor growth and xenograft experiments (SK-BR-3) as indicated. All experiments were conducted in compliance with the NIH Guides for the Care and Use of Laboratory Animals and approved by the University of Miami Institutional Animal Care and Use Committee.

Construction, Expression, and Characterization of αHER2-huEndo Fusion Protein

The human endostatin (huEndo) gene was cloned from the human collagen, type XVIII, alpha 1 gene by PCR using primers 5'-

CCCCTCGCGATATCACAGCCACCGCGACTTCCAGCCG-3' and 5'-CCCCGAATTCGTTAACCCTTGGAGGCAGTCATGAAGC-3'. PCR products were subcloned into pCR-Blunt II-TOPO vector and sequenced. A single-point mutant clone at a position 125 in wild-type human endostatin was derived by site-directed mutagenesis using PCR with phosphorylated primer, 5'p-GGCTCGGACGCCAACGGGCGC-3'. An alanine residue was substituted for proline at position 125 by site-directed mutagenesis. A point mutation in human endostatin at position 125 (proline to alanine; huEndo-P125A) has been reported to enhance endothelial cell binding and anti-angiogenic activity. The subcloned huEndo and huEndo-P125A genes were ligated in frame to the carboxyl end of the heavy chain constant domain of human IgG3 in the vector pAT135 (24). The endostatin heavy chain constant region was joined to the anti-HER2 variable region of a recombinant humanized monoclonal antibody 4D5-8 (trastuzumab; Genentech, San Francisco, CA) in the expression vector (pSV2-his) containing HisD gene for eukaryotic selection (25, 26).

To obtain active endostatin fusion proteins, the αHER2-huEndo fusion constructs were stably transfected into SP2/0 or P3X63Ag8.653 myeloma cells expressing the anti-HER2 kappa light chain by electroporation as described previously (27). The αHER2-huEndo fusion proteins were biosynthetically labeled with [^{35}S]methionine (Amersham Biosciences, Piscataway, NJ), immunoprecipitated using IgGSorb suspension (S. aureus cells), and analyzed by SDS-PAGE. The endostatin fusion proteins were purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma, St. Louis, MO) (27).

Flow cytometry

To detect the binding of αHER2-huEndo fusion proteins to HER2 antigen, human breast cancer cells, SK-BR-3, or murine mammary tumor cells, EMT6 and EMT6-HER2, were incubated at 4°C with 1μg/ml of endostatin fusion proteins, αHER2 IgG3, or isotype control. After 15 min the cells were washed with PBS containing 0.1% BSA and 0.05% NaN3, and the bound fusion proteins were identified with either FITC conjugated anti-human IgG-, or the endostatin domain was recognized with biotinylated anti-human endostatin antibody and secondarily stained with a streptavidin-PE conjugate at 4°C. After incubation, the cells were washed twice and resuspended in 0.4 ml of PBS. FACScan flow cytometer was used for data acquisition. Background staining was estimated after incubation with the secondary FITC or PE labeled antibody alone.

Matrigel Tube Formation Assay

The matrigel tube formation assay was performed in 48-well plates, as previously reported (28, 29). Each well of pre-chilled 48-well cell culture plates was coated with 100 μl of unpolymerized Matrigel (7 mg/ml) and incubated at 37 °C for 30-45 minutes. HUVECs were harvested with trypsin, and 4 x 10^4 cells were resuspended in 300 μL of full endothelial cell growth medium (see above) and treated with endostatin, control antibody or the various αHER2-huEndo fusion proteins before plating onto the Matrigel-coated plates. After 16 hours of incubation, endothelial cell tube formation was assessed with an inverted photomicroscope, and microphotographs of the center of each well were taken at low power (40x). Tube formation by untreated HUVECs in full endothelial cell growth medium was used as a control.

HUVEC Proliferation Assay

A total of 4 x 10^3 cells in 100 µl of the endothelial basal medium with 1% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) were placed into each well of a 96-well plate, treated with αHER2-huEndo fusion proteins and controls, and incubated at 37 °C for 72 hours; control cells were cultured in basal medium, 1% FBS, and antibiotics, as above (28, 29). VEGF (10 ng/ml) or bFGF (10 ng/ml) were added as stimulants of endothelial cell proliferation. After the 72-hour incubation, WST-1 (10 µl, Roche, Indianapolis, IN) was added to each well, and after a 3-hour incubation at 37 °C, absorbance at 450 nm was determined for each well with a microplate reader (Bio-Rad Laboratories, Hercules, CA). Data presented are the average of triplicate experiments which were repeated twice.

***In vivo* Tumor Growth Assays:**

To evaluate ant-tumor activity of αHER2-huEndo fusion proteins SK-BR-3 cells (2 x 10^6 per mouse) were implanted s.c. in the flank of SCID mice (23). On day 5, mice (5 per group) were injected *i.v.* with equimolar amounts of purified αHER2-huEndo fusion proteins (42 µg), αHER2 IgG3 (34 µg), or human endostatin (8 µg). This treatment was repeated every other day for 13 doses. Tumor size was measured with calipers and growth rates were recorded and calculated using the following equation: tumor volume (mm^3) = 4/3 x 3.14 x {(long axis + short axis)/4}^{3}.

Murine mammary tumor EMT6 cells were transduced with a retroviral vector encoding human HER2 antigen as described previously (23). The EMT6-HER2 cells that were used in these studies proliferate at the same rate *in vitro* as parental EMT6 cells (data not shown) (23).

The *in vivo* anti-tumor efficacy and specificity of αHER2-huEndo fusion proteins was examined using the EMT6 and EMT6-HER2 cell lines simultaneously implanted contralaterally in syngeneic BALB/c mice. To determine efficacy of αHER2-huEndo fusion proteins, BALB/c mice (3-8 per group, 4–6 weeks) were injected *s.c.* with 1 x 10^6 EMT6-HER2 cells in the right flank and/or control EMT6 cells in the left flank. On day 6, mice were injected *i.v.* with the αHER2-huEndo fusion proteins (42 μg/injection, 2 x 10^{-10} mol, equimolar to 8 μg human endostatin), αHER2 IgG3 alone (34 μg/injection, 2 x 10^{-10} mol), endostatin alone (8 μg/injection, 4 x 10^{-10} mol), or PBS as a control. All mice received a total of eleven injections at 2-day intervals and tumor growth analyzed as described above.

Immunofluorescent staining

To investigate blood vessel formation in tumors treated with αHER2-huEndo-P125A fusion protein, EMT6 and EMT6-HER2 cell lines (1 x 10^6) were contralaterally implanted in syngeneic BALB/c mice (n = 4) as described above (23). On day 4, mice were injected *i.v.* with αHER2-huEndo-P125A (42 μg/injection), or PBS as a control every two days. On day 12, two mice were sacrificed for analysis of vascularity after four treatments. On day 18, another two mice were sacrificed for analysis after seven treatments. Tumors were excised from the sacrificed mice and frozen in liquid nitrogen and the 8 μm frozen sections were prepared. The tumor sections were fixed with methanol for 10min, washed with PBS 3 times, and incubated with blocking solution for 1 hour in a humidified chamber. Slides were then washed with PBS 3 times, for 10 min. For immunofluorescent staining, diluted primary antibodies (anti-CD31 antibody conjugated with biotin: 1:200 (BD, Franklin Lakes, NJ)) in PBS were added to each slide. After incubation at room temperature overnight, the sections were incubated with diluted secondary antibodies

conjugated with Alexa Fluor 488 (1:500, (Invitrogen, Carlsbad, CA)) with PBS, and then with diluted DAPI (1:5000, (Molecular Probes, Carlsbad, CA) in a humidified chamber and mounted with Gel mounting media (Biomeda Corp. Foster City, CA). The stained images were analyzed with a Zeiss microscope.

Statistical Analysis

Statistical analysis for HUVEC proliferation assays and tumor growth curves was performed using a two tailed, paired Student's t test. Differences were considered statistically significant at $P < 0.05$.

Results

Construction and purification of αHER2-huEndo fusion proteins

We previously demonstrated enhanced anti-tumor activity of an anti-HER2 antibody-mEndo fusion protein, relative to that seen with anti-HER2 antibody and/or murine endostatin delivered alone or in combination. In preparation for potential human use and an effort to reduce antigenicity, we sought to "humanize" the fusion protein through substitution of human endostatin sequences for the murine endostatin fusion domain. The human endostatin (huEndo) gene was cloned from the human collagen, type XVIII, alpha 1 gene by PCR. Clones containing wild-type human endostatin were identified. A point mutation in human endostatin at position 125 (proline to alanine; huEndo-P125A) within a previously mapped angiogenic domain of endostatin has been reported to have increased endothelial cell binding and enhance anti-angiogenic activity (19-21). We also introduced the P125A mutation into human endostatin using site directed mutagenesis. The subcloned huEndo and huEndo-P125A genes were ligated in frame to the carboxyl end of the heavy chain constant domain of human IgG3 and the endostatin heavy chain constant region was then joined to the anti-HER2 variable region derived from the humanized monoclonal antibody 4D5-8 (HER2, trastuzumab; Genentech) in the expression vector (pSV2-his) containing HisD gene for eukaryotic selection. A schematic of the resulting fusion proteins is shown in Fig. 1A.

The anti-HER2 IgG3-huEndo fusion protein constructs were then stably transfected into SP2/0 or P3X63Ag8.653 myeloma cells stably expressing the anti-HER2 kappa light chain in order to assemble the entire anti-HER2 IgG3-huEndo fusion proteins, anti-HER2 IgG3-huEndo (αHER2-huEndo) and anti-HER2 IgG3-huEndo-P125A (αHER2-huEndo-P125A) (Fig. 1A).

The αHER2-huEndo fusion proteins were biosynthetically labeled with [^{35}S]methionine and analyzed by SDS-PAGE. αHER2-huEndo fusion proteins of the expected molecular weight were secreted as the fully assembled H_2L_2 form (Fig.1B). The secreted [^{35}S]methionine-labeled proteins had a molecular weight of ~220 kDa under non-reducing conditions, the size expected for a complete antibody (170 kDa) with two molecules of endostatin (25 kDa each) attached (Fig. 1A). Following reduction, heavy and light chains of the expected molecular weight were observed (85 kDa and 25 kDa, respectively) (Fig. 1B). The endostatin fusion proteins were then purified from culture supernatants using a protein A column.

Binding ability of anti-HER2 human endostatin fusion proteins to HER2 target antigen and to HUVECs

We investigated whether the endostatin fusion proteins could recognize the HER2 antigen (Fig. 2). The HER2 expressing human breast cancer cell line, SK-BR-3, and murine mammary tumor cells, EMT6 and EMT6-HER2 were used to test binding to HER2 antigen, Using anti-human IgG antibody as a detection antibody, αHER2 IgG3, αHER2-huEndo and αHER2-huEndo-P125A, bound to the HER2+ SKBR3 breast cancer cells and EMT6-HER2 cells (Fig. 2-I and 2-II, respectively), while the isotype control antibody (anti-dansyl IgG3) did not bind to SK-BR-3 and EMT6-HER2. αHER2 IgG3, αHER2-huEndo or αHER2-huEndo-P125A did not bind to parental EMT6 cells that did not express any HER2 antigen (Fig. 2-III).

To investigate structural integrity of the human endostatin moiety of the fusion proteins, anti-HER2 human endostatin fusion proteins were incubated with SK-BR-3, EMT6-HER2 and EMT6. The human endostatin domain of fusion proteins bound to SK-BR-3 and EMT6-HER2 was detected with biotinylated anti-human endostatin and stained with streptoavidin-PE conjugate.

αHER2-huEndo and αHER2-huEndo-P125A were both recognized following binding to SK-BR-3 and EMT6-HER2 by the anti-human endostatin detection antibody (Fig. 2-IV and 2-V), while αHER2 IgG3 and the isotype control antibodies were not detected. Endostatin could be detected using either αHER2-huEndo or αHER2-huEndo-P125A as the primary antibody and both fusion proteins bound to HER2+ SK-BR-3 and EMT6-HER2 cells with similar affinity.

To determine whether αHER2-huEndo fusion proteins could bind to endothelial cells, HUVECs were treated with αHER2 IgG3, huEndo, or αHER2-huEndo fusion proteins, and human endostatin domain bound to HUVECs was detected with biotinylated anti-human endostatin and stained with streptoavidin-PE conjugate. Binding of human endostatin and αHER2-huEndo fusion proteins to HUVECs was readily detected, while the isotype control, or αHER2 IgG3 binding was not detected (Fig. 2-VI). Of note αHER2-huEndo-P125A showed slightly greater binding to HUVECs relative to either human endostatin or αHER2-huEndo.

Inhibition of endothelial tube formation by the αHER2-huEndo fusion proteins

To evaluate anti-angiogenic properties of the human endostatin antibody fusion proteins, we tested the effects of the αHER2-huEndo fusion proteins in an *in vitro* angiogenesis assay in which human endothelial cells are plated on Matrigel, and spontaneously aggregate and assemble into multicellular capillary-like tubular structures in response to vascular stimuli (*e.g.* bFGF, VEGF, FBS) (28, 29). Neither parental antibody nor human endostatin alone showed appreciable inhibition of tube formation. In contrast, αHER2-huEndo fusion protein treatment strongly inhibited assembly into tubular structures, with cells remaining dispersed and exhibiting a morphology resembling adherent cells on plastic (scattered phenotype) in a dose dependent fashion (Fig. 3A). This phenomenon has been previously reported with the NC1 domain of

collagen XVIII and oligomeric forms of endostatin (17, 18). The αHER2-huEndo and αHER2-huEndo-P125A fusion proteins showed significantly greater inhibition of HUVEC tube formation compared to αHER2 IgG3 or to human endostatin. The increased *in vitro* anti-angiogenic effect of αHER2-huEndo fusions relative to native endostatin may be due to presentation of endostatin as a dimer as previously reported (17, 18).

Yokoyama and colleagues have reported that a mutant version of endostatin in which the proline at 125 is substituted with an alanine, showed greater anti-angiogenic activity than native endostatin *in vitro* (19-21). Inhibition of tubule assembly seen with αHER2-huEndo-P125A was significantly greater than that seen for αHER2-huEndo at comparable concentrations (Fig. 3A-IV to 3A-IX) and treatment of HUVEC at 45 nM resulted in complete disruption of tubule formation and extensive morphologic changes (scatter) (Fig. 3A-VIII, IX). Mutation of proline to alanine at amino acid position 125 of human endostatin in the fusion protein therefore increased inhibition of tubule formation by endothelial cells compared to either native endostatin (huEndo) or wild type endostatin fusion protein (αHER2-huEndo).

Proliferation of endothelial cells by the αHER2-huEndo fusion proteins

We next assessed the effects of αHER2-huEndo fusion proteins on endothelial cell (EC) proliferation. HUVECs were exposed to increasing concentrations of the fusion proteins for 72 hrs in the absence or presence of either VEGF or bFGF. Both wild type and mutant antibody-endostatin fusion proteins markedly inhibited endothelial cell proliferation induced by either VEGF (Fig. 3B-I) or bFGF (Fig. 3B-II). HUVEC proliferation was more effectively inhibited by αHER2-huEndo-P125A at comparable concentrations than by αHER2-huEndo or by endostatin alone (Fig. 3B).

Anti-tumor efficacy in human breast cancer SK-BR-3 xenografts

SK-BR-3 is a HER2-amplified human breast cancer cell line which grows slowly in SCID mice. Trastuzumab, anti-HER2 IgG1, is able to inhibit the growth of human breast cancer SK-BR-3 overexpressing HER2 alone or in combination with chemotherapy. We assayed for anti-tumor activity of αHER2-huEndo fusion proteins against human breast cancer SK-BR-3 xenografts in SCID mice. A representative experiment is shown in Figure 4. Equimolar doses of protein were injected every other day for 4 weeks (Fig. 4). In Fig. 4A, endostatin and αHER2 IgG3 moderately inhibited tumor growth relative to the non-treated group (PBS, *p* value = 0.012, 0.005, respectively) by day 29, while treatment with αHER2-huEndo and αHER2-huEndo-P125A resulted in markedly greater inhibition of growth (*p* value = 0.011, 0.009, respectively). Treatment with αHER2-huEndo-P125A completely eradicated tumors after 30 days and showed the highest degree of inhibition. The proportion of tumor-free survivors was higher for the αHER2-huEndo-P125A group (5 of 5 in the experiment shown compared to PBS (0 of 5), αHER2 IgG3 and human endostatin (1 of 5), and αHER2-huEndo (2 of 5) (Fig.4B). Mice treated with αHER2-huEndo-P125A showed improved survival relative to those treated with αHER2-huEndo, human endostatin alone, or αHER2 IgG3 alone (Fig. 4B). Similar results were seen in multiple experiments of which a representative experiment is shown.

Anti-tumor efficacy requires presence of the HER2 target

In a prior study, we demonstrated that the *in vivo* targeting of anti-angiogenic proteins using αHER2-murine endostatin fusion protein preferentially inhibited tumor growth of tumors expressing HER2, compared to contralaterally implanted parental tumor (no HER2 expression)

(23). To investigate whether the ability of αHER2-huEndo-P125A fusion protein to specifically target HER2 expressing tumors enhanced efficacy, BALB/c mice were simultaneously implanted with EMT6 and EMT6-HER2 tumors on opposite flanks. Mice were then treated with either αHER2-huEndo-P125A or human endostatin. Equimolar administration of αHER2-huEndo-P125A to mice showed preferential growth inhibition of EMT6-HER2, when compared to parental EMT6 implanted on the contralateral flank (Fig. 5A). αHER2-huEndo-P125A (Fig 5A-II and 5A-V) inhibited EMT6-HER2 tumor growth more effectively than PBS (Fig. 5A-I and 5A-IV, *p* value = 0.003), or endostatin (Fig. 5A-III and 5A-VI, *p* value = 0.003). Individual tumor volume on day 16 was compared as the slope (*m*) between EMT6 and EMT6-HER2 tumors in paired tumors within mice (Fig.5B). αHER2-huEndo-P125A ($m = -1029.88 \pm 135.69$) showed greater difference between EMT6 and EMT6-HER2 tumors in paired tumors than endostatin ($m = -291.11 \pm 238.45$, *p* value = 0.033) and PBS ($m = -140.69 \pm 123.63$, *p* value = 0.002). . Selective targeting of HER2 expressing tumor was therefore required for maximum efficacy.

Immunofluorescent staining of blood vessel of treated tumor

To investigate the effects of αHER2-huEndo-P125A fusion protein on tumor angiogenesis tumors were resected , histologic sections of tumors were derived from treated and untreated mice after 4 or 7 treatments, and tumor microvasculature was visualized using anti-PECAM fluorescence immunostaining (Fig. 6). Immunofluorescent staining of EMT6-HER2 tumors demonstrated that the antibody-endostatin fusion treated group showed thin, short, and fragmented blood vessels on day 12 after 4 treatments (Fig. 6-VI to 6-VIII), compared to those of the PBS treated group (Fig. 6-II to 6-IV). By day 18 (7 treatments), EMT6-HER2 tumor from

one of two mice treated with αHER2-huEndo-P125A had completely regressed and the other in the treated group demonstrated very small tumor without any clearly stainable vessels, while vasculature was readily demonstrated in PBS treated tumors (data not shown).

Discussion

Anti-angiogenic therapy with endostatin has been shown to block tumor growth in mice with little or no evidence for emergence of resistance despite multiple cycles of therapy, in a variety of murine models. In several murine models, repeated treatment with endostatin resulted in permanent eradication of tumors (1, 2, 8). However, Phase I/II studies of human endostatin did not demonstrate the levels of anti-tumor activity seen in murine models, although these clinical trials proved that human endostatin is a very safe drug when used at a variety of dose schedules (9-13). We hypothesized that several of the logistical disadvantages of the long-term treatment with high dosages of endostatin could be overcome if the half-life of endostatin could be extended and if endostatin could be specifically targeted to the tumor, to achieve higher local concentrations and greater specificity. In addition, we hypothesized that endostatin might be more effective if delivered as a dimer in the context of an antibody fusion protein (17, 18). We had previously demonstrated that an anti-HER2 IgG3-C_H3-murine endostatin fusion protein retained anti-angiogenic activity, exhibited prolonged serum half-life and stability, selectively targeted tumors bearing HER2, inhibited blood vessel formation, and inhibited tumor growth more effectively *in vivo* than either endostatin or anti-HER2 antibody alone or delivered in combination (23). We demonstrated the ability of such fusions to selectively localize to HER2+ tumors, and noted enhanced efficacy in several murine models including the CT26-HER2, EMT6-HER2 murine tumors which had been engineered to express human HER2, and against SKBR3 xenografts that constitutively express high levels of HER2 (23).

In order to reduce the possible antigenicity of the murine endostatin fusion domain in preparation for human application, we have now constructed two new fusions based on human endostatin and on a mutated form of endostatin with increased anti-angiogenic properties. The

αHER2-huEndo and αHER2-huEndo-P125A fusion proteins markedly inhibited endothelial tube formation and proliferation of HUVEC *in vitro*, and did so more efficiently than human endostatin. The αHER2-huEndo-P125A fusion protein showed greater inhibition of tube formation *in vitro* than either native endostatin or than wild type αHER2-huEndo fusion. Treatment of established SK-BR-3 xenografts in SCID mice with the αHER2-huEndo-P125A fusion resulted in greater inhibition of growth, compared to αHER2 IgG3, human endostatin, or αHER2-huEndo fusion protein treated mice. The αHER2-huEndo fusion protein specifically targeted tumors expressing HER2 and inhibited tumor growth in syngeneic mice simultaneously implanted with EMT6 and EMT6-HER2. . αHER2-huEndo-P125A inhibited EMT6-HER2 tumor growth more effectively than PBS, or human endostatin (p value = 0.003). Combining the targeting capability of anti-HER2 antibody with the anti-angiogenic activity of human endostatin presented in a dimer form in the context of a fusion antibody improves the inhibition of endothelial tube formation and proliferation of HUVEC *in vitro* and enhances anti-tumor activity *in vivo*.

In the endothelial tube formation experiment, the human endostatin fusion proteins led to profound morphologic changes in HUVEC, and prevented tube formation. It has been reported that human or murine endostatin treatment inhibited HUVEC assembly into tubular structures *in vitro*, with cells remaining dispersed and exhibiting a morphology resembling adherent cells on plastic rather than aggregating into characteristic capillary-like tubes (17, 18). Dimers or trimers of endostatin stimulated the motility of endothelial cells, but endostatin monomers did not, which demonstrated that endostatin oligomerization was required for the efficient inhibition of tube formation activity (17, 18). Since the αHER2-huEndo fusion proteins retain two endostatin domains in a fusion protein, they may effectively present endostatin as a dimer, and this may

result in more dispersed and scattered morphology of HUVECs seen in these experiments. Dimerization of the endostatin domain of the fusion proteins could in theory further facilitate binding to integrins, perleacan, and glypicans, and further increasing fusion protein activity. Introduction of a point mutation into human endostatin at position 125 (proline to alanine; huEndo-P125A) has been reported to enhance endothelial cell binding, anti-angiogenic activity, and anti-tumor activity (19-21). The mutant αHER2-huEndo-P125A fusion variant inhibited tube formation of HUVEC *in vitro* and tumor growth *in vivo* more effectively than αHER2-huEndo. Four synthetic peptides corresponding to the sequences 6–49 (I), 50–92 (II), 93–133 (III), and 134–178 (IV) of human endostatin have been examined for their ability to inhibit endothelial cell proliferation, migration, and both *in vitro* and *in vivo* angiogenesis (30, 31). Fragment I was found to be fully anti-angiogenic in all of the angiogenesis assays, and sometimes showed even greater potency and efficacy than full-length human endostatin itself. Unexpectedly, fragment III exhibited a pro-angiogenic activity, increased endothelial cell migration, and produced neovascularization to an extent similar to that seen for vascular endothelial growth factor, and enhanced the angiogenic response to vascular endothelial growth factor in a corneal pocket assay. The P125A point mutation is located within fragment III, and may lead a conformational change that reduces the pro-angiogenic properties of fragment III. Furthermore, human endostatin has an internal Asn-Gly-Arg (NGR) motif at position 126–128 following the proline at position 125 (20). Asn-Gly-Arg containing motifs have been shown to target tumor vasculature. Mutation of the 125-proline may therefore affect vascular targeting by NGR motif. P125A endostatin bound to endothelial cells more efficiently than wild type endostatin and exhibited greater inhibition of both proliferation and migration of endothelial cells (20). P125A endostatin also localized into tumor tissue,more efficiently and displayed greater inhibition of

growth of colon cancer in athymic mice, and also decreased expression of key pro-angiogenic growth factors (20). Vascular endothelial growth factor and angiopoietin 1 were downregulated more by P125A endostatin than by native endostatin (20). These results suggested that an antibody fusion based on the P125A endostatin mutant, αHER2-huEndo-P125A, might inhibit tumor growth *in vivo* more effectively than αHER2-huEndo. This proved to be the case in our experiments.

Antigenicity of the fusion proteins is a theoretical problem which may affect activity *in vivo* in either immunocompetent mice or potentially in man, and reduce efficacy. Antigenicity in an immunocompetent mouse may not predict antigenicity in humans. There are several examples of fusions in approved clinical use or in clinical trials in which antigenicity has not been a major problem: Anti-TNFR-fusions (32, 33), DAB_{389}IL-2 (ONTAK) in patients with lymphoid leukemias and lymphoma (34), VEGFR-fusion (VEGF-Trap in patients with solid tumors and non-Hodgkin's lymphomas) (35). Therefore the antigenicity of human endostatin fusion protein and the effects of antigenicity on efficacy cannot be predicted *a priori* from mouse models. We saw enhanced efficacy in both xenograft models, in which T and B cells were absent, and in immunocompetent mice suggesting that antigenicity may not abrogate efficacy at least in the murine models we tested. Whether antigenicity of the fusions will affect efficacy in man needs to be directly tested.

Linking endostatin to an antibody may significantly enhance the anti-tumor activity of trastuzumab (23). Because the overall response rates of HER2+ breast cancers to trastuzumab remain relatively low (15–34%) (35-38), this approach holds promise for increasing both response rate and duration relative to trastuzumab, and may expand the spectrum of anti-tumor activity of trastuzumab given alone or in combination with other anti-tumor strategies such as

other cytotoxic agents (carboplatin, docetaxel) (39-42), and/or anti-angiogenic drugs (e.g. bevacizumab; anti-VEGF antibody, thrombospondin-1) (43-46). Since administration of endostatin appears to be quite safe, antibody-endostatin fusion proteins may also be suitable for use in the adjuvant setting as well. Indeed, we have observed marked synergy when the anti-HER2 IgG3-murine endostatin fusion and the anti-VEGF antibody bevacizumab were given in combination to SK-BR-3 xenograft containing mice (data not shown). This suggests the antibody fusion may be particularly useful when combined with other anti-angiogenic approaches.

In addition to endostatin, other anti-angiogenic domains could also theoretically be incorporated into fusions (*e.g*. angiostatin, tumstatin, etc). Since endostatin is known to be a powerful and global regulator of angiogenic gene expression, we concentrated initial experiments on endostatin as a candidate fusion. Finally, in addition to the HER2 antigenic target, targeting anti-angiogenic proteins using antibody is a versatile approach that could be applied to other tumor targets (such as epidermal growth factor receptor or prostate-specific membrane antigen) through substitution with other antibody specificities/variable domains. This approach could in theory be used to enhance efficacy and utility of antibodies directed to tumor antigens in which parental antibody shows only modest efficacy (*e.g.* Cetuximab) (47-49).

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Figure Legends

Fig. 1. Schematic diagram of anti-HER2 IgG3-human endostatin fusion proteins. Endostatin domain in orange (A) and expression of anti-HER2 IgG3-C_H3-endostatin fusion proteins (wild type and the mutant type P125A) (B). Secreted human endostatin fusion proteins were labeled with [^{35}S]methionine and immunoprecipitated with Protein A and analyzed under non-reducing and reducing conditions. Anti-HER2 IgG3-C_H3-murine endostatin fusion was used as a control.

Fig. 2. Binding of anti-HER2 human endostatin fusion proteins to HER2 antigen and HUVECs, and recognition by anti-human endostatin antibody. Human breast cancer cells, SK-BR-3 (I, IV), murine mammary tumor cells, EMT6-HER2 (II, V) and EMT6 (III), and HUVECs (VI) were incubated with αHER2-huEndo (thin black line, filled with red), αHER2-huEndo-P125A (thick black line, unfilled), αHER2 IgG3 (thick green line, unfilled), human endostatin (thick blue line), or isotype control (anti-dansyl IgG3, thin black line, filled with gray). The unfilled, thin black line is unstained (the secondary reagents only). The bound fusion proteins were identified with either anti-human IgG-FITC conjugated (I-III), or recognized with biotinylated anti-human endostatin antibody and secondarily stained with a streptavidin-PE conjugate (IV-VI).

Fig. 3. Effects of anti-HER2 IgG3-huEndo fusion proteins on EC tube formation and EC proliferation. A. HUVECs (4 x 10^4 cells) were resuspended in 300 μl of full endothelial cell growth medium and treated with the various αHER2-huEndo fusion proteins before plating onto the Matrigel-coated plates. After 16-20 hr of incubation, tube formation was observed through an

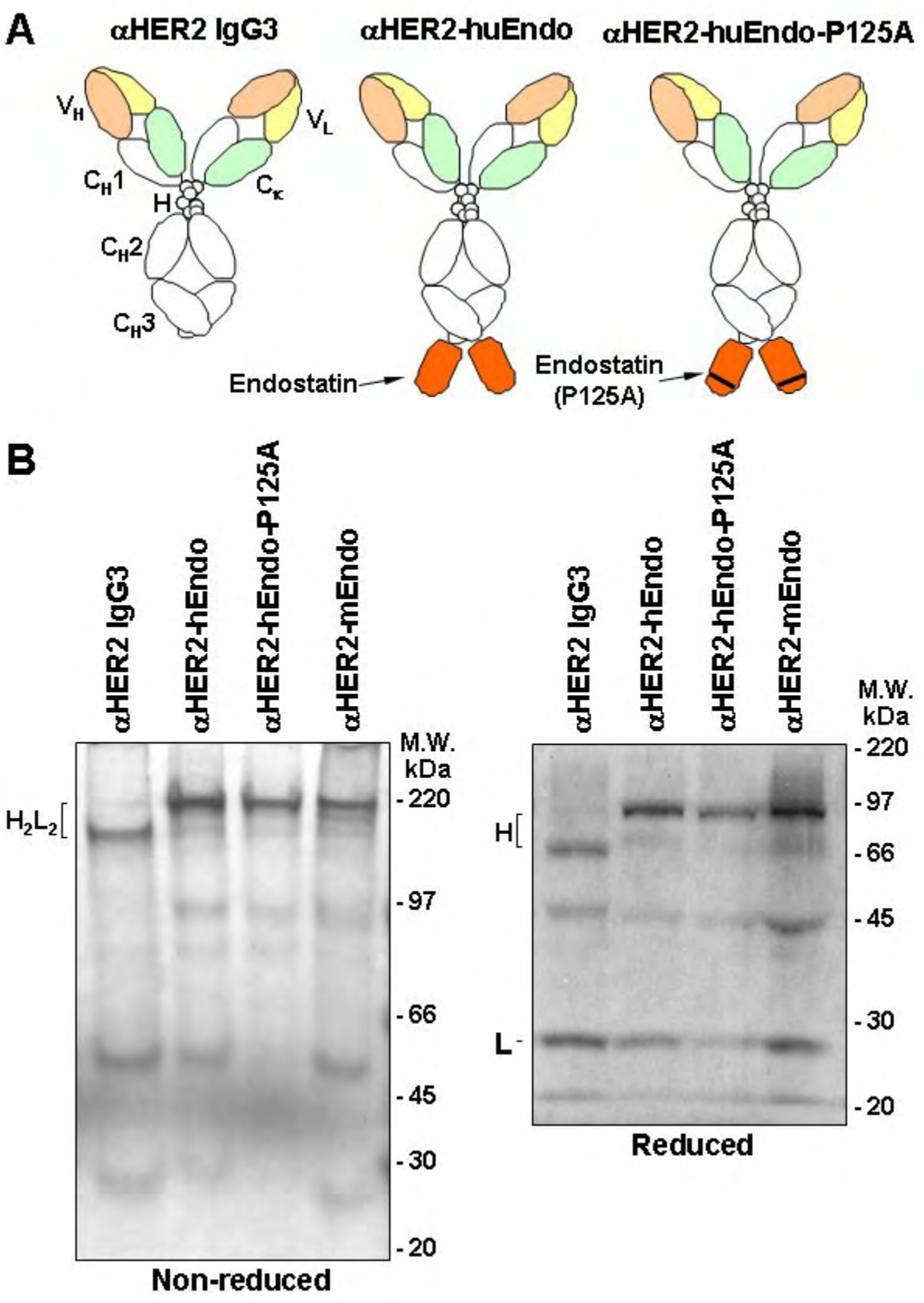
inverted photomicroscope. Full media was used as negative control (I). Tube formation with αHER2 IgG3 (II. 45.46 nM) and huEndo (III. 45.46 nM) were compared to those with αHER2-huEndo (IV-VI, 4.55, 22.73, 45.46 nM, respectively) and αHER2-huEndo-P125A (VII-IX, 4.55, 22.73, 45.46 nM, respectively). Experiments repeated at least twice. B. HUVECs (4×10^3 cells) were treated with increasing concentrations of the endostatin fusion proteins and proliferation measured at 72 hrs. I. VEGF: HUVEC proliferation induced by VEGF (10 ng/ml). II. FGF: HUVEC proliferation induced by bFGF (10 ng/ml). The data are presented as the mean of triplicate determinations ± SD. Experiments were repeated twice.

Fig. 4. Anti-tumor efficacy of anti-HER2 IgG3-huEndo fusion proteins. SCID mice (n=5) were *s.c.* inoculated with $2x10^6$ SK-BR-3 on the right flank back on day 0, then the mice were *i.v.* injected with anti-HER2 IgG3-huEndo fusion proteins (42 μg), antiHER2 IgG3 (34.9 μg), human endostatin (8 μg), or PBS every other day (indicated with arrow starting on day 5). (A) Tumor growth was measured with calipers. Tumor volume was calculated as 4/3 x 3.14 x $\{(\text{long axis} + \text{short axis})/4\}^3$. The values represent mean ± SEM of tumor volume (mm^3) of 5 mice. (B) Survival of mice per treatment group. Mice with greater than 2000 mm^3 tumor volume were euthanized.

Fig. 5. Anti-tumor activity of αHER2-huEndo fusion-P125A protein in a syngeneic mouse model. BALB/c mice (n=3-8 per group) were implanted *s.c.* contralaterally with EMT6 (I-III) and EMT6-HER2 (IV-VI) ($1x10^6$ cells per mouse), followed on day 6 by equimolar injections every other day (11 times) of αHER2-huEndo-P125A (42 μg), human endostatin (8 μg), or PBS.

A. Individual tumor measurements of mice treated with αHER2-huEndo-P125A fusion protein are presented. B. Comparison of tumor growth between untargeted and targeted tumors on day 16. EMT6 and EMT6-HER2 tumor measurements of individual mice are paired and presented. The thick red lines represent average measurements.

Fig. 6. Analysis of tumor vascularity. BALB/c mice (n=4 per group) were implanted *s.c.* contralaterally with EMT6 and EMT6-HER2 ($1x10^6$ cells per mouse), followed on day 4 by equimolar injections every other day (7 time treatments) of αHER2-huEndo-P125A (42 μg), or PBS. On day 12, two mice were sacrificed for the blood vessel analysis after four treatments. Histologic sections of tumors from the sacrificed mice were analyzed using immunofluorescent staining for PECAM (II-IV, VI-VIII; green color). DAPI (I, III, V, VII; blue color) was used for counter-staining of the nucleus. Representative immunofluorescent staining of EMT6-HER2 tumors treated with PBS (I-IV) or αHER2-huEndo-P125A (V-VIII) is presented. Magnification: 50x (I-III, V-VII) or 100x (IV, VIII). Bars: 500 μm for I-III and V-VII, 200 μm for IV and VIII.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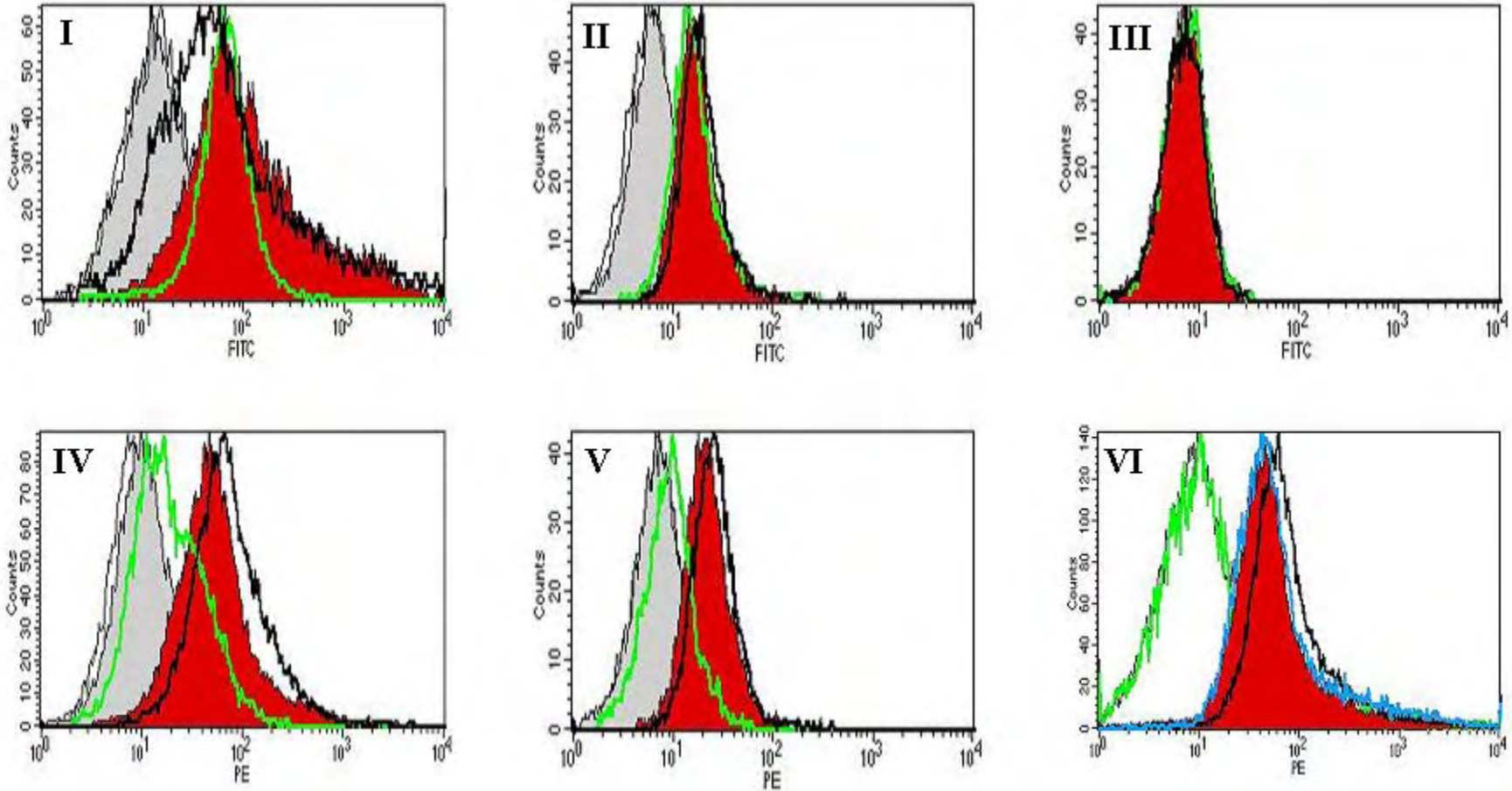
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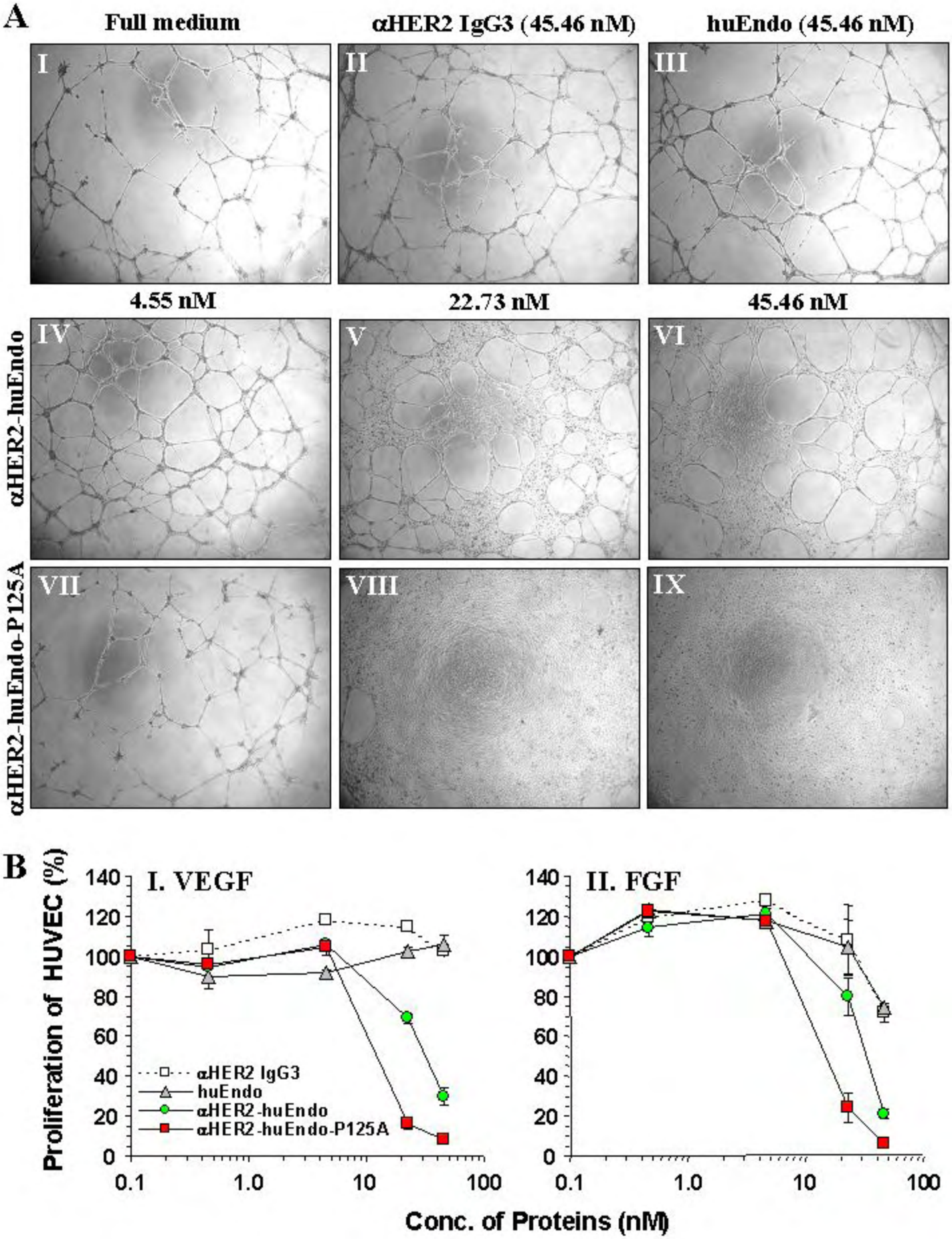
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II
III
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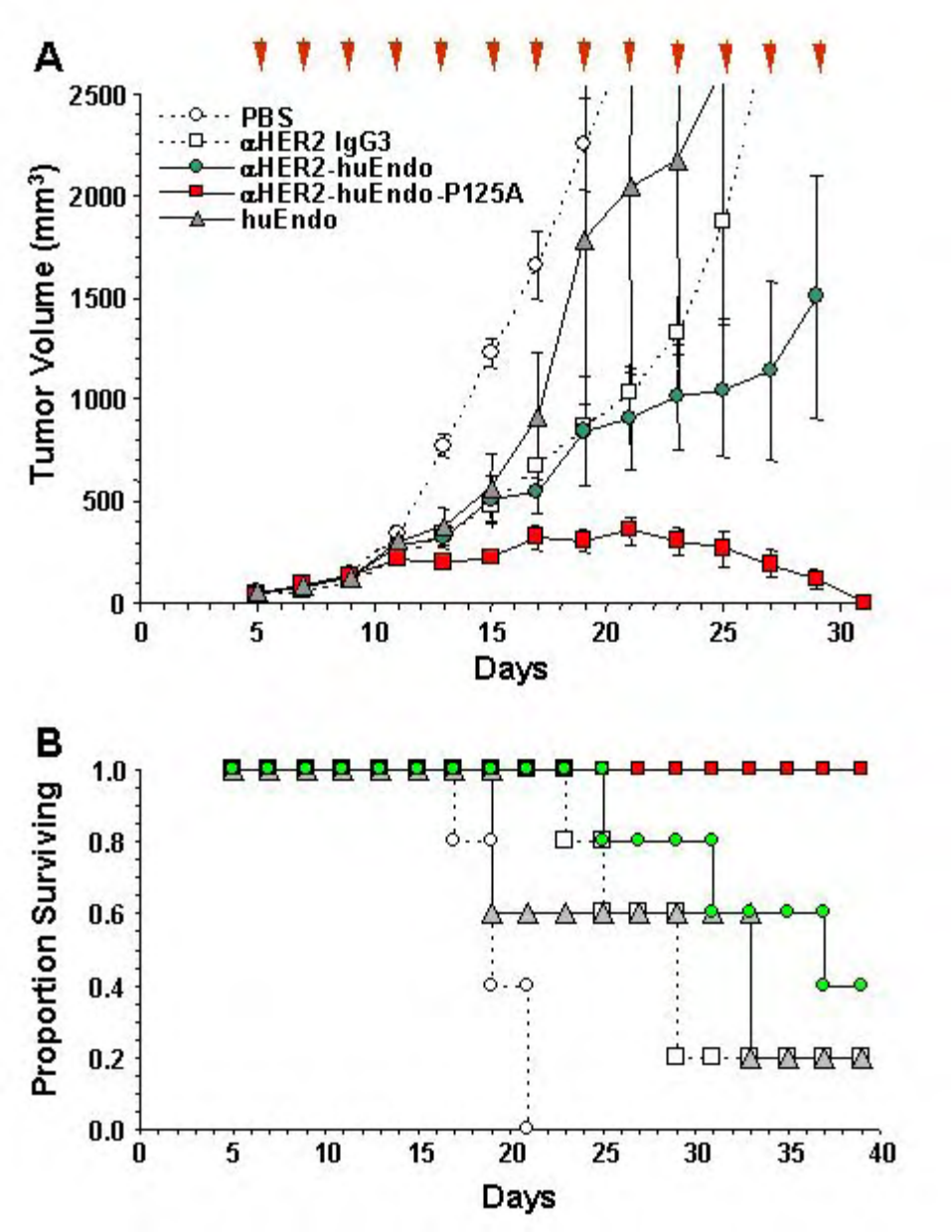
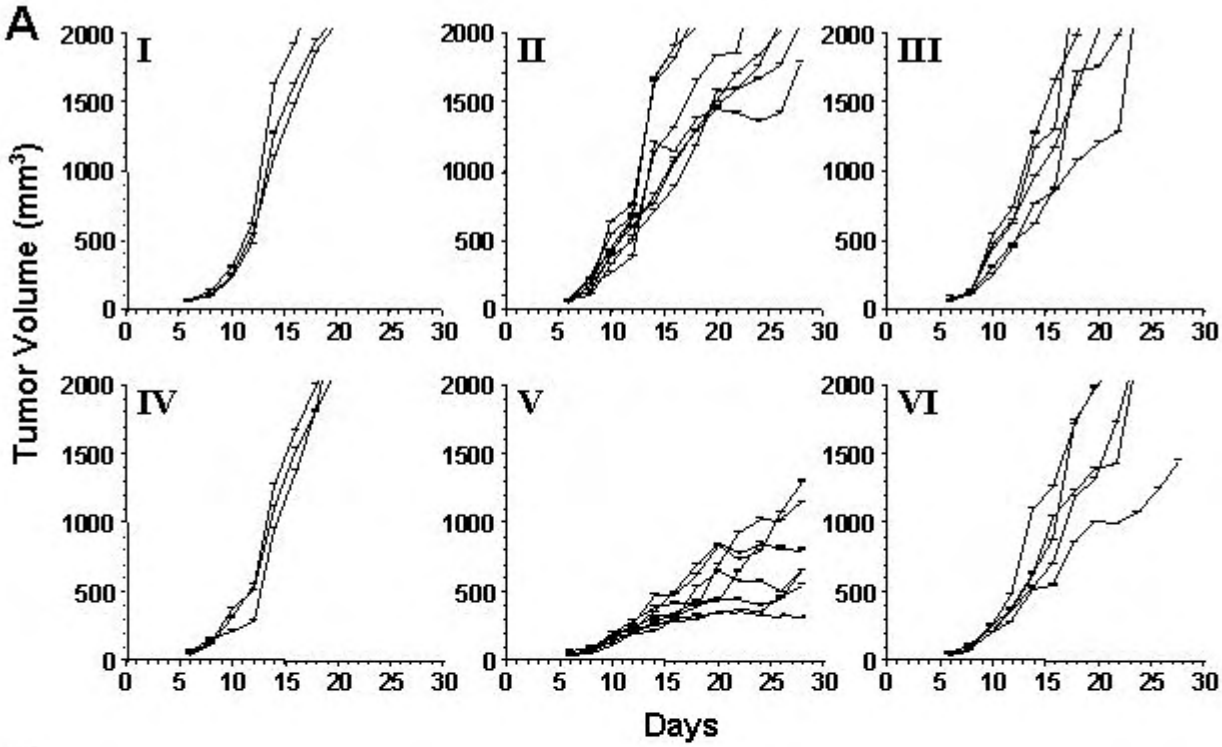
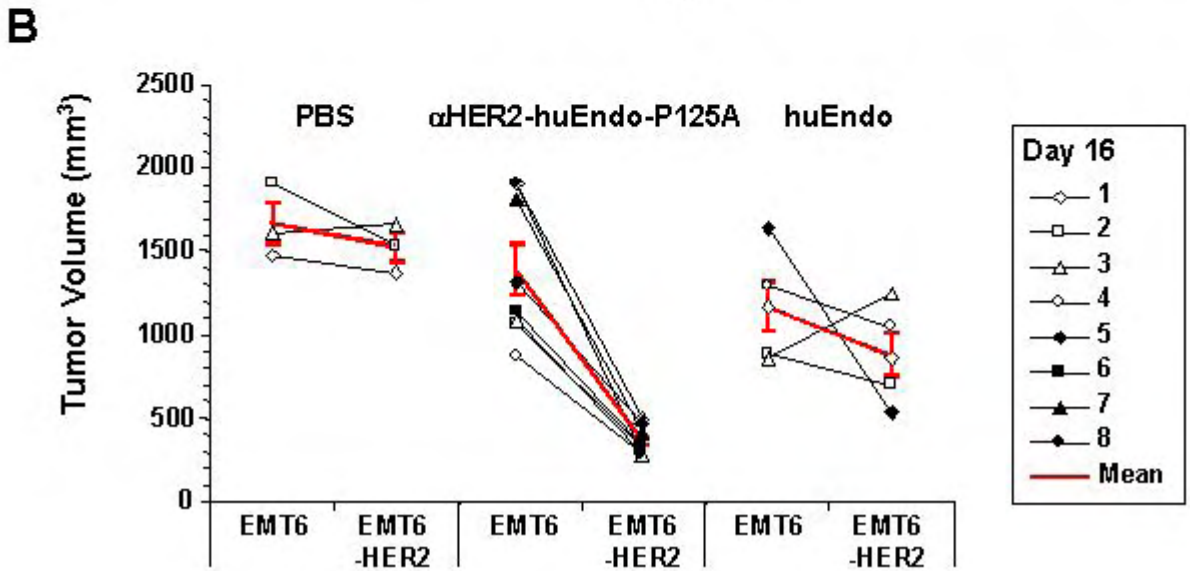
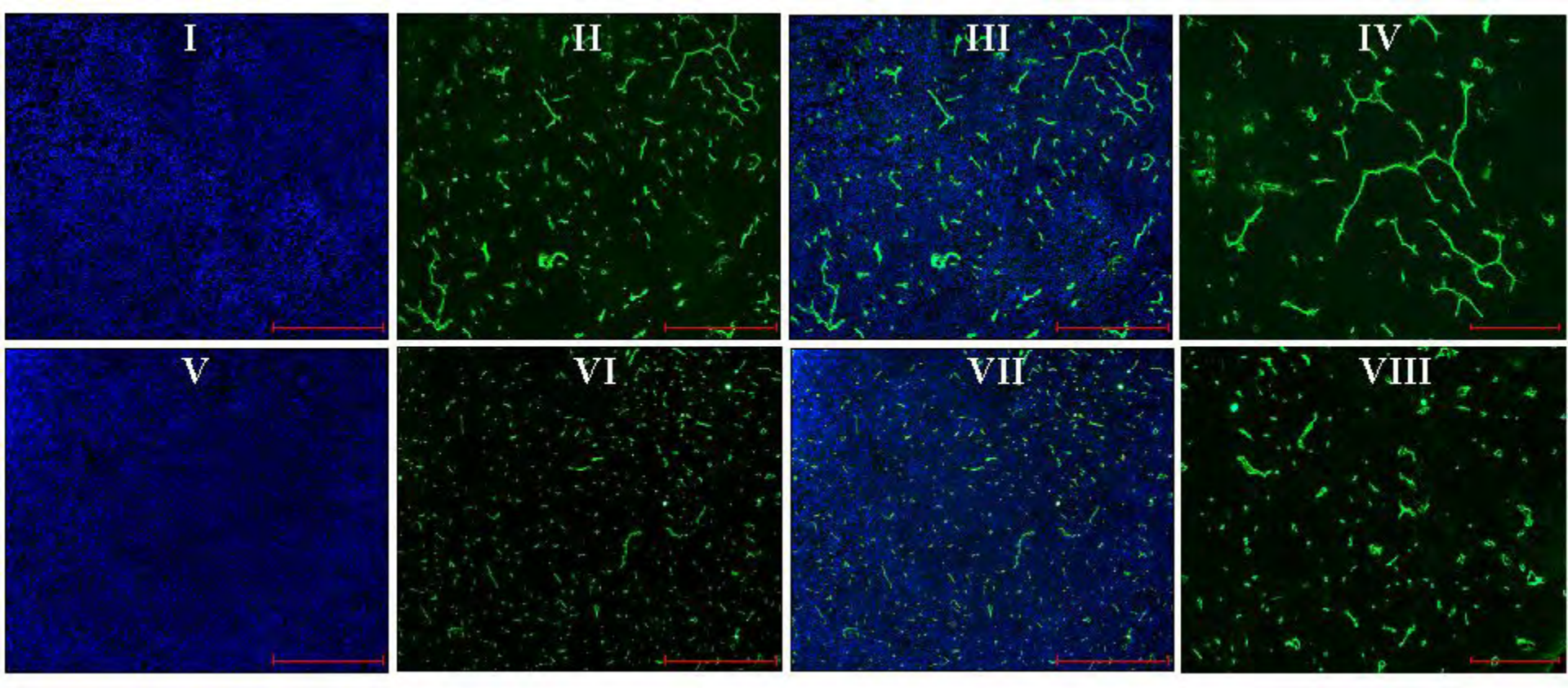
22.73 nM
45.46 nM
αHER2-huEndo
IV
V
VI
αHER2-huEndo-P125A
VII
VIII
IX
B
I. VEGF
II. FGF
Proliferation of HUVEC (%)
αHER2 IgG3
huEndo
αHER2-huEndo
αHER2-huEndo-P125A
Conc. of Proteins (nM)


A
PBS
αHER2 IgG3
αHER2-huEndo
αHER2-huEndo-P125A
huEndo
Tumor Volume (mm3)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Days
B
Proportion Surviving
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
Days


A
I
II
III
IV
V
VI
Tumor Volume (mm3)
Days
0
500
1000
1500
2000
0
5
10
15
20
25
30


B
Tumor Volume (mm3)
PBS
αHER2-huEndo-P125A
huEndo
EMT6
EMT6 -HER2
Day 16
1
2
3
4
5
6
7
8
Mean
0
500
1000
1500
2000
2500


I
II
III
IV
V
VI
VII
VIII

Shin, Seung-uon

From: support@abstractsonline.com
Sent: Tuesday, February 05, 2008 10:17 PM
To: Shin, Seung-uon
Subject: 2008 AACR Annual Meeting in San Diego, CA - Poster Abstract #6564

February 5, 2008

Re: 2008 AACR Annual Meeting in San Diego, CA
Temporary Abstract Number #6564
Title: Targeted delivery of an anti-HER2 antibody-human endostatin P125A mutant fusion protein results in enhanced anti-tumor activity in murine and human breast tumor models.

Dear Dr. Shin:

Your above-referenced abstract has been scheduled for presentation in a Poster Session at the 2008 AACR Annual Meeting in San Diego, CA and will be published in the 2008 Proceedings of the American Association for Cancer Research. Presentation information pertaining to your abstract is below:

Session ID: Tumor Biology 9
Session Date and Time: Sunday, April 13, 2008, 1:00 PM
Permanent Abstract Number: 1105

Please refer to the printed Final Program (distributed onsite) or the online Annual Meeting Itinerary Planner [available in mid-March through the AACR Website at http://www.aacr.org] for the exact location of your presentation.

Instructions for Presenters in Poster Sessions can be found on the 2008 AACR Annual Meeting home page:
www.aacr.org/page12435.aspx

Poster Session presenters at the AACR Annual Meeting must register for the full meeting at the rate appropriate to their membership status and obtain their own hotel accommodations. Registration and housing information are included below:

Advance Registration Deadline: March 7, 2008
Online Registration
http://www.aacr.org/home/scientists/meetings--workshops/annual-meeting-2008/registration.aspx

Housing Deadline: March 5, 2008
Online Housing System
http://www.aacr.org/home/scientists/meetings--workshops/annual-meeting-2008/accommodations.aspx

Online Travel Information and Reservation System
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For more information, visit the 2008 AACR Annual Meeting home page at:
http://www.aacr.org/home/scientists/meetings--workshops/annual-meeting-2008.aspx

Thank you for your participation in the 2008 AACR Annual Meeting.

Sincerely,
Eileen P. White, Ph.D.

Program Committee Chairperson

PLEASE NOTE: This document is your official notice of acceptance. No separate letter of acceptance will be mailed.

AACR ANNUAL MEETING 2008
April 12-16, 2008 • San Diego Convention Center • San Diego, CA

Category: **Tumor Biology 9**

Session Title: **Inhibition Of Angiogenesis 1**

#1105 Targeted delivery of an anti-HER2 antibody-human endostatin P125A mutant fusion protein results in enhanced anti-tumor activity in murine and human breast tumor models. Seung-Uon Shin, Hyun-Mi Cho, Jaime Merchan, Jin Zhang, Joseph D. Rosenblatt. Univ. of Miami, Sylvester Cancer Ctr., Miami, FL.

The antiangiogenic protein endostatin has shown considerable anti-tumor activity in animal models. However, only modest anti-tumor activity has been observed in human trials. Trastuzumab as a single agent has activity in HER2+ breast cancer when used alone or in combination with chemotherapy. We previously constructed an anti-HER2 antibody-murine endostatin fusion which demonstrated enhanced anti-tumor activity compared to anti-HER2 antibody or endostatin given alone or administered in combination. Antibody targeting is designed to enhance local delivery of endostatin to tumor as well as increase endostatin half-life. In addition, the presentation of endostatin as a dimer due to fusion with each heavy chain is designed to further augment activity.

We have constructed several anti-HER2 *human* endostatin fusion proteins by fusing human endostatin to the 3' end of a humanized anti-HER2 IgG3 antibody. Two anti-HER2-antibody endostatin fusion proteins were generated using fusion with native human endostatin (αHER2-huEndo) or with a mutant endostatin containing a P125A substitution (αHER2-huEndo-P125A) which has been reported to confer increased antiangiogenic activity by Yokoyama *et. al*. Native and huEndo-P125A antibody fusion proteins markedly inhibited endothelial vasculogenesis in a tube formation assay and the proliferation of HUVEC *in vitro*, and did so more efficiently than human endostatin at equimolar concentrations. αHER2-huEndo-P125A showed markedly greater inhibition of vasculogenesis *in vitro* than either native endostatin or αHER2-huEndo fusion protein. Treatment of established SKBR-3 xenografts in SCID mice with the αHER2-huEndo-P125A fusion resulted in complete tumor regression and greater inhibition of growth, compared to either anti-HER2 IgG3, human endostatin, or αHER2-huEndo treated mice ($p = 0.023$, 0.008, 0.004, respectively). Both αHER2-huEndo and αHER2-huEndo-P125A specifically targeted tumors expressing HER2 in syngeneic mice simultaneously implanted with murine mammary tumor cell line EMT6 and EMT6 tumor engineered to express the HER2 antigen (EMT6-HER2) on opposite flanks. αHER2-huEndo-P125A inhibited EMT6-HER2 tumor growth more effectively than PBS ($p = 0.003$), huEndo ($p = 0.003$), or αHER2-huEndo ($p = 0.004$).

Linking endostatin to an antibody may significantly enhance anti-tumor activity of both trastuzumab and endostatin. Mutant anti-HER2 huEndo-P125A fusion protein showed markedly enhanced anti-tumor activity. Targeting endostatin may improve anti-tumor activity relative to trastuzumab and/or endostatin delivered alone or in combination Antibody-endostatin fusion represents a versatile approach that could also be applied to other tumor targets with alternative antibody specificities.

Citation Format
Shin S, Cho H, Merchan J, Zhang J, Rosenblatt JD. Targeted delivery of an anti-HER2 antibody-human endostatin P125A mutant fusion protein results in enhanced anti-tumor activity in murine and human breast tumor models [abstract]. In: Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA. Philadelphia (PA): AACR; 2008. Abstract nr 1105.

Targeted delivery of an anti-HER2 antibody-human endostatin P125A mutant fusion protein results in enhanced anti-tumor activity in murine and human breast tumor models

Seung-Uon Shin, Hyun-Mi Cho, Jaime Merchan, Jin Zhang, and Joseph D. Rosenblatt

Hematology-Oncology/Microbiology and Immunology, University of Miami/ Sylvester Comprehensive Cancer Center

Abstract

The antiangiogenic protein endostatin has shown considerable anti-tumor activity in animal models. However, only modest anti-tumor activity has been observed in human trials. Trastuzumab as a single agent has activity in HER2+ breast cancer when used alone or in combination with chemotherapy. We previously constructed an anti-HER2 antibody-murine endostatin fusion which demonstrated enhanced anti-tumor activity compared to anti-HER2 antibody or endostatin given alone or administered in combination. Antibody targeting is designed to enhance local delivery of endostatin to tumor as well as increase endostatin half-life. In addition, the presentation of endostatin as a dimer due to fusion with each heavy chain is designed to further augment activity.

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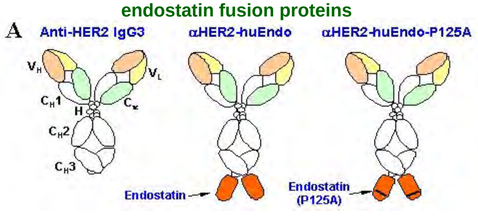
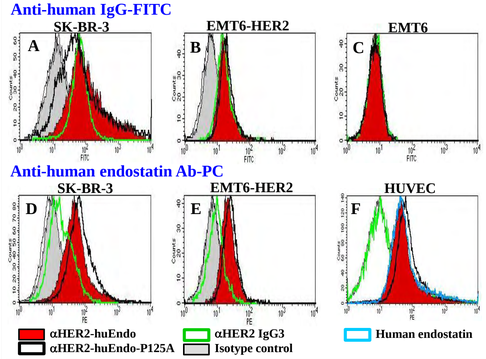
Linking endostatin to an antibody may significantly enhance anti-tumor activity of both trastuzumab and endostatin. Mutant anti-HER2 huEndo-P125A fusion protein showed markedly enhanced anti-tumor activity. Targeting endostatin may improve anti-tumor activity relative to trastuzumab and/or endostatin delivered alone or in combination Antibody-endostatin fusion represents a versatile approach that could also be applied to other tumor targets with alternative antibody specificities.

Results

Schematic diagram of anti-HER2 IgG3-human endostatin fusion proteins

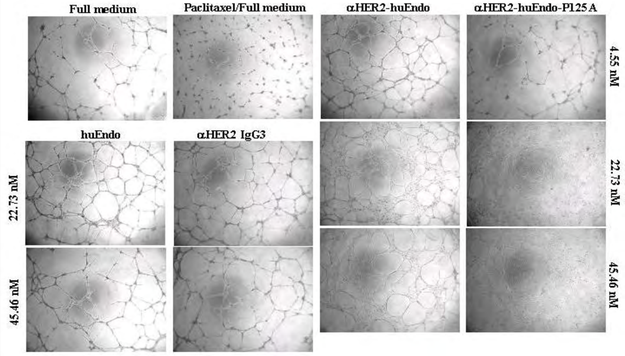


Binding of anti-HER2 human endostatin fusion proteins



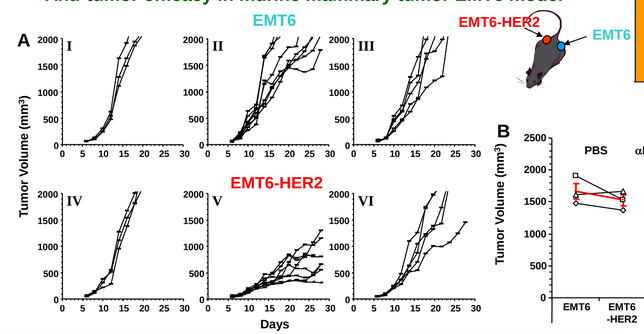
Endostatin could be detected using either αHER2-huEndo or αHER2-huEndo-P125A as the primary antibody and both fusion proteins bound to HER2+ SK-BR-3 and EMT6-HER2 cells with similar affinity, while αHER2 IgG3 and the isotype control antibodies were not detected.
Binding of human endostatin and αHER2-huEndo fusion proteins to HUVECs was readily detected, while the isotype control, or αHER2 IgG3 binding was not detected. Of note αHER2-huEndo-P125A showed slightly greater binding to HUVECs relative to either human endostatin or αHER2-huEndo.

Effects on HUVEC Tube Formation



Neither parental antibody nor human endostatin alone showed appreciable inhibition of tube formation. In contrast, αHER2-huEndo fusion protein treatment strongly inhibited assembly into tubular structures, with cells remaining dispersed and exhibiting a morphology resembling adherent cells on plastic (scattered phenotype) in a dose dependent fashion. This phenomenon has been previously reported with the NC1 domain of collagen XVIII and oligomeric forms of endostatin. The αHER2-huEndo and αHER2-huEndo-P125A fusion proteins showed significantly greater inhibition of HUVEC tube formation compared to αHER2 IgG3 or to human endostatin. The increased *in vitro* anti-angiogenic effect of αHER2-huEndo fusions relative to native endostatin may be due to presentation of endostatin as a dimer as previously reported.

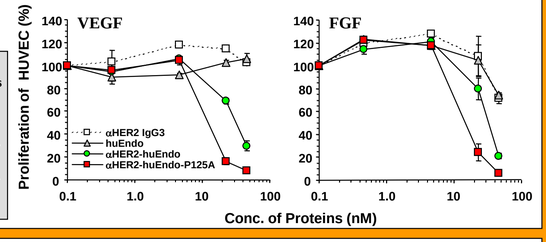
Anti-tumor efficacy in murine mammary tumor EMT6 model



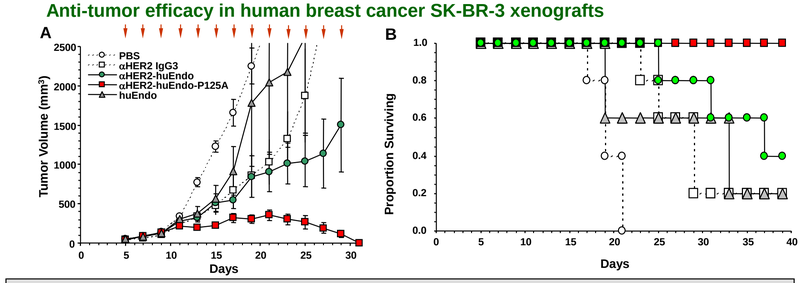
Equimolar administration of αHER2-huEndo-P125A to mice showed preferential growth inhibition of EMT6-HER2, when compared to parental EMT6 implanted on the contralateral flank (A). αHER2-huEndo-P125A (II and V) inhibited EMT6-HER2 tumor growth more effectively than PBS (I and IV, p value = 0.003), or endostatin (III and VI, p value = 0.003). Individual tumor volume on day 16 was compared as the slope (m) between EMT6 and EMT6-HER2 tumors in paired tumors within mice (B). αHER2-huEndo-P125A (m = -1029.88 ± 135.69) showed greater difference between EMT6 and EMT6-HER2 tumors in paired tumors than endostatin (m = -291.11 ± 238.45, p value = 0.033) and PBS (m = -140.69 ± 123.63, p value = 0.002). Selective targeting of HER2 expressing tumor was therefore required for maximum efficacy.

Proliferation of HUVEC

HUVECs were exposed to increasing concentrations of the fusion proteins for 72 hrs in the absence or presence of either VEGF or bFGF. Both wild type and mutant antibody-endostatin fusion proteins markedly inhibited endothelial cell proliferation induced by either VEGF or bFGF. HUVEC proliferation was more effectively inhibited by αHER2-huEndo-P125A at comparable concentrations than by αHER2-huEndo or by endostatin alone.

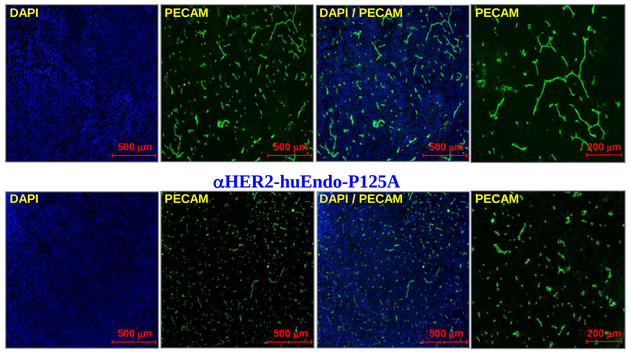


Anti-tumor efficacy in human breast cancer SK-BR-3 xenografts



(A) Endostatin and αHER2 IgG3 moderately inhibited tumor growth relative to the non-treated group (PBS, p value = 0.012, 0.005, respectively) by day 29, while treatment with αHER2-huEndo and αHER2-huEndo-P125A resulted in markedly greater inhibition of growth (p value = 0.011, 0.009, respectively). Treatment with αHER2-huEndo-P125A completely eradicated tumors after 30 days and showed the highest degree of inhibition. (B) The proportion of tumor-free survivors was higher for the αHER2-huEndo-P125A group (5 of 5 in the experiment shown compared to PBS (0 of 5), αHER2 IgG3 and human endostatin (1 of 5), and αHER2-huEndo (2 of 5). Mice treated with αHER2-huEndo-P125A showed improved survival relative to those treated with αHER2-huEndo, human endostatin alone, or αHER2 IgG3 alone.

Tumor Vascularity



To investigate the effects of αHER2-huEndo-P125A fusion protein on tumor angiogenesis tumors were resected, histologic sections of tumors were derived from treated and untreated mice after 4 or 7 treatments, and tumor microvasculature was visualized using anti-PECAM fluorescence immunostaining. Immunofluorescent staining of EMT6-HER2 tumors demonstrated that the antibody-endostatin fusion treated group showed thin, short, and fragmented blood vessels on day 12 after 4 treatments, compared to those of the PBS treated group. By day 18 (7 treatments), EMT6-HER2 tumor from one of two mice treated with αHER2-huEndo-P125A had completely regressed and the other in the treated group demonstrated very small tumor without any clearly stainable vessels, while vasculature was readily demonstrated in PBS treated tumors.

Discussion

Treatment of established SK-BR-3 xenografts in SCID mice with the αHER2-huEndo-P125A fusion resulted in greater inhibition of growth, compared to αHER2 IgG3, human endostatin, or αHER2-huEndo fusion protein treated mice. The αHER2-huEndo fusion protein specifically targeted tumors expressing HER2 and inhibited tumor growth in syngeneic mice simultaneously implanted with EMT6 and EMT6-HER2. αHER2-huEndo-P125A inhibited EMT6-HER2 tumor growth more effectively than PBS, or human endostatin (p value = 0.003).

The αHER2-huEndo and αHER2-huEndo-P125A fusion proteins markedly inhibited endothelial tube formation and proliferation of HUVEC *in vitro*, and did so more efficiently than human endostatin. The αHER2-huEndo-P125A fusion protein showed greater inhibition of tube formation *in vitro* than either native endostatin or than wild type αHER2-huEndo fusion. Since the αHER2-huEndo fusion proteins retain two endostatin domains in a fusion protein, they may effectively present endostatin as a dimer, and this may result in more dispersed and scattered morphology of HUVECs seen in these experiments.

Combining the targeting capability of anti-HER2 antibody with the anti-angiogenic activity of human endostatin presented in a dimer form in the context of a fusion antibody improves the inhibition of endothelial tube formation and proliferation of HUVEC *in vitro* and enhances anti-tumor activity *in vivo*.

Research Funded by the U.S. Army Medical Research and Material Command under W81XWH-05-1-0351 Grant